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TRANSFER AND REPLICATION SYSTEMS OF THE INCHI1 PLASMID R27

BY

PETER J. NEWNHAM

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A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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P. Newnham

c/o Dept. of Medical
Microbiology and Immunology
Edmonton, Alberta
T6G 2H7

DATE: June 14/95

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled Transfer and Replication Systems of the IncHI1 Plasmid R27 submitted by Peter J. Newnham in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Dr. John Bell

Dr. John Elliott

Dr. Laura Frost

Dr. Bob Iyer (External Examiner)

Dr. Diane Taylor (Supervisor)

Date: 8 June 1995

This thesis is dedicated to my wife Elizabeth,

my parents Gail and Roger, and my grandmother Helen,

whose constant encouragement have kept me going.

ABSTRACT

R27 is a member of the HI incompatibility complex of plasmids, whose unique characteristics include their large size, narrow host range, and thermosensitive conjugation system. A minimal replicon was cloned from R27 and its nucleotide sequence determined. Sequence data and replicative properties suggested it to be distantly related to the iteron-controlled replicons of several other plasmid incompatibility groups. This replicon was designated RepHI1A.

A second PolI-independent replicon (RepHI1B) was located on the R27 map, along with an incompatibility determinant of unknown function. These determinants, along with the RepHI1A replicon, map within a 15-kb region of the plasmid and represent maintenance determinants unique to IncHI1 plasmids. Stability experiments suggested that the incompatibility determinant may be involved in partitioning, or some other stabilizing function.

A transfer complementation system was established whereby the IncHII plasmid pHH1508a was used to complement various transfer-deficient mutants of R27. The degree of complementation observed varied considerably from mutant to mutant, and also exhibited considerable host-specific contribution.

Effects of sudden temperature shifts on R27 conjugation efficiency were examined. Transient shifts from permissive to restrictive temperatures produced rapid and lasting reductions in transfer frequency for subsequent conjugation tests, while shifts during a one hour mating completely repressed transfer if carried out within the first five minutes.

Examination of the conjugative pili produced by HI1 and HI2 plasmids showed the pili to be composed of a single pilin monomer of 3.0 - 3.2-kDa. Polyclonal antisera to purified H pili was used to monitor the expression of pilin protein at various temperatures, which demonstrated that pilin is only synthesized in detectable quantities under temperatures favorable to transfer.

A transfer-derepressed derivative of R27 was mutagenized with a mini-Tn10 transposon to produce numerous transfer-deficient mutants. Insertion sites were mapped and shown to fall in two broad regions of the plasmid, Tra1 and Tra2. Although numerous insertions reduced or eliminated sensitivity to H-specific phages, few had any noticeable effect on pilin synthesis. Nucleotide sequence data from the S*al*I-F region of R27, revealed the presence of numerous open reading frames, several of which possess homology with Tra proteins of the F plasmid.

These studies have enhanced our understanding of H plasmid genetics and shown this medically important group of antibiotic resistance-bearing

elements to be distantly related to those of numerous other incompatibility groups.

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ABBREVIATIONS

ATP	adenosine triphosphate
BHI	brain heart infusion
BSA	bovine serum albumin
DNA	deoxyribonucleic acid
DTE	dithioerythritol
DTT	dithiothreitol
EDTA	disodium ethylene diamine tetraacetate
ELISA	enzyme-linked immunosorbent assay
IHF	integration host factor
kb	kilobase
kbp	kilobase pairs
kDa	kilodalton
LB	Luria broth
M	molar
ml	millilitre
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PBS	phosphate buffered saline

RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
SSC	standard sodium citrate
TBS	tris buffered saline
TCA	trichloroacetic acid
TE	Tris EDTA
Tris	Tris (hydroxymethyl) aminomethane
TSA	trypticase soy agar
TSB	trypticase soy broth

Chapter I Introduction

A. Plasmids

Plasmids are extrachromosomal deoxyribonucleic acid elements that are carried by many procaryotes. By definition, a plasmid is not essential to the survival of its host, but often confers a selective advantage to the host by way of genetic information it carries. Hundreds of phenotypes have been associated with the carriage of plasmids, ranging from antibiotic resistance to complete biochemical pathways for the synthesis or degradation of a variety of compounds.

Although different topological forms have been discovered (55), most procaryotic plasmids exist as double-stranded circular DNA molecules. Plasmids as small as two to three kilobase pairs (kbp) to greater than 1000 kbp in size have been characterized, demonstrating enormous diversity in size. Similarly, plasmid copy number ranges considerably from as little as one copy per chromosome equivalent to hundreds of copies per chromosome equivalent.

Nearly all bacterial genera have been shown to carry plasmid molecules. This observation underscores the important roles plasmids play in natural settings, as well as an amazing ability for self-preservation, considering the parasitic relationship a plasmid has with its host organism.

In fact, many plasmids possess elaborate mechanisms to ensure their survival and maintenance within host organisms.

Numerous plasmids encode conjugation systems that provide a mechanism of replicative transmission from one host cell to another. Conjugation provides a means of plasmid dissemination within a bacterial population. In addition, various plasmid groups are capable of transferring to and replicating within a broad range of bacterial genera. These broad-host-range plasmids have been the subject of intense study in recent years.

The study of bacterial plasmids has provided a wealth of knowledge, much of which has seen practical application in the field of plasmid vector construction for use in molecular biology and biotechnology, as well as other areas.

B. Incompatibility

Related plasmids cannot stably coexist within the same bacterial host cell without undergoing some form of recombinational event. In the absence of selective pressure, related plasmids will tend to segregate within the bacterial population. This phenomenon is known as incompatibility and has been used as a method of plasmid classification. Plasmids that demonstrate an incompatibility reaction with one another are classified as members of the same incompatibility group. Using this method, at least 25 (29, 145)

different incompatibility groups have been assigned to plasmids that are predominant in gram negative organisms. In addition, 13 incompatibility groups differentiate the numerous Pseudomonad plasmids.

Although incompatibility is an observable phenomenon, it is not the result of genes whose primary action is to effect incompatibility. Rather, it is typically a consequence of two plasmids sharing a related replication, partitioning, or other maintenance system (105). Under these circumstances, the common maintenance mechanism shared by the plasmids cannot distinguish between them, resulting in instability of one or both plasmids. So-called *inc* loci are typically so named because incompatibility is an easily observable trait, but further investigation usually reveals the loci in question to be involved in some aspect of plasmid maintenance.

C. Replication

Plasmid replication takes many forms, but the most common in gram negative organisms is a theta style replication system similar to that of the chromosome. Plasmids of gram positive organisms more typically employ rolling circle replication schemes. Most plasmids rely heavily upon the replication machinery of the host organism, with only one or two components encoded by the plasmid itself. Initiation events are usually controlled by

plasmid-encoded components in the replication process, which in turn controls plasmid copy number.

At a very basic level, plasmid replicons found in gram negative organisms can be divided into two general groups, based on regulation of replication initiation events (105). The first group, known as the inhibitor-target type, negatively regulates replication initiation events by the production of an inhibitor molecule that interacts with (either directly or indirectly) a component of the replication system to suppress initiation. The second group, known as iteron-controlled replicons, possess multiple repeated oligonucleotide sequences to which an essential replication initiator binds.

1. Inhibitor-target control of replication

The best known example of this type of replication control is plasmid ColE1 and its relatives (p15A, pMB1, CloDF13, and others). Replication is initiated in this plasmid by the production of a long primer RNA molecule, known as RNA II. This RNA forms a persistent hybrid with its DNA template in the origin of replication. After processing by RNase H, the RNA is utilized as a primer by DNA PolI, which leads to replication of the entire plasmid (59, 127). No plasmid-encoded proteins are required for replication - the plasmid is entirely dependent on host components.

The inhibitory molecule in this case is a small countertranscript RNA known as RNA I. This RNA molecule is antisense to RNA II, and binds to

the 5' region of RNA II, disrupting its secondary structure, which prevents proper priming at the origin of replication (89, 156). The rate of synthesis and degradation of RNA I directly affects the rate of initiation of replication at the origin, and thus copy number of the plasmid.

Plasmids of the IncFII group also possess replicons that employ an inhibitor-target scheme for replication control. In these cases, replication is initiated by an essential initiator protein, known as RepA. This is in contrast with ColE1 and its relatives, which rely entirely on host proteins to initiate replication. The inhibitor molecule is a small antisense RNA (CopA) that binds to the 5' end of the *repA* mRNA (104). Interaction of CopA with the *repA* mRNA prevents the translation of a small leader peptide (*tapA*) whose translation is required for translation of the RepA protein (18, 174). Hence, interaction of CopA with its target acts to indirectly inhibit RepA synthesis at the level of translation.

Plasmids of the I incompatibility complex ($IncI_1$, $IncI_\gamma$, $IncI_\delta$, IncB, IncK, and IncZ) have replicons that are similar in structure to those of the IncFII plasmids. Plasmids pMU720 (IncB) and ColIb-P9 ($IncI_1$) are the best studied examples of this class. As with the IncFII replicon, the replicons of pMU720 and ColIb-P9 encode an essential replication initiator protein (RepA or RepZ) whose expression is inhibited posttranscriptionally by an inhibitor RNA molecule (RNAI) (12, 114). In order for synthesis of the RepA protein to occur, a small leader peptide (*repB* or *repY*) must be produced, and a tertiary

structure, known as a pseudoknot, must form in the *repA/repZ* mRNA (171). Interaction of RNAI with its target prevents translation of the mRNA for the leader peptide, and formation of the pseudoknot.

2. Iteron control of replication

Iteron-controlled replicons are considerably more complex in terms of control of replication initiation events. Plasmids of many different incompatibility groups possess replicons of this basic type. A common theme is the presence of a gene encoding an essential replication initiator protein, and a series of repeated oligonucleotides, termed iterons (105), to which the replication initiator binds. The mechanism by which initiation events are controlled is not clearly understood at this time. A simple titration model was initially proposed, in which Rep protein bound to iterons was unavailable for initiation, and only when Rep protein exceeded a critical level would a new round of replication initiate (158). However, this model failed to explain a number of observations. For example, the presence of excess Rep protein fails to significantly increase plasmid copy number (108). Hence, other models were proposed in an attempt to explain this anomalous behaviour. Currently, it is thought that Rep protein, bound to iteron sequences, causes a "handcuffing" or "looping" reaction (either intra- or inter-molecular) that effectively inhibits replication initiation by steric hindrance of the origin (103). Other levels of control, such as auto-regulation of the Rep protein, and the conversion of Rep protein from an inactive to an active form, may also

contribute to the overall rate of replication and hence, plasmid copy number.

Plasmid pSC101 was originally studied as a paradigm for this type of replication control. The minimal replicon of this oligo-copy plasmid (6-8 copies per chromosome equivalent) consists of a gene encoding a *trans*-acting replication initiator (RepA), and a *cis*-acting site, the origin of replication (87). The RepA protein is autoregulated, and binds both to sites overlapping its own promoter, and to iterons in the origin itself. In addition, the origin harbors binding sites for DnaA and IHF, and an AT-rich region.

Other replicons employing the iteron-binding mechanism of replication control include those from plasmids of incompatibility groups IncF (98, 111), IncT (66), IncY (3), IncX (38) and the broad host range plasmids of incompatibility groups IncN (69), IncP (133), and IncQ (58). It should be noted that the replicons of broad host range plasmids are in most cases considerably more complex than those of narrow host range plasmids, and often encode multiple Rep proteins, or possess multiple origins of replication. These features likely enable these replicons to replicate in a wider variety of host organisms than would otherwise be possible.

D. Stability

Most plasmids possess loci that increase their stability. Since plasmids impose a metabolic burden on their host organisms, plasmid-free

segregants tend to grow more rapidly and would slowly come to dominate a population of bacterial cells, in the absence of positive selection. As a result, many plasmids go to great lengths to ensure that plasmid-free segregants appear and proliferate at very low frequency, thereby ensuring the stable inheritance of the plasmid from one generation to the next.

One mechanism used by some plasmids to ensure stable inheritance is to replicate to a relatively high copy number. This dramatically reduces the likelihood that a daughter cell will arise in a division event without at least one copy of the plasmid, assuming completely random segregation. For example, the plasmid ColE1 and its close relatives replicate to a copy number of approximately 20 per chromosome equivalent. Assuming completely random segregation amongst daughter cells, the probability of a plasmid-free segregant arising with this copy number is approximately 5×10^{-5} (2^{-19}). Of course, plasmids with lower copy number have significantly higher probabilities of generating plasmid-free segregants, in the absence of non-random segregation. A plasmid with a copy number of 1-2 (such as F, P1, or the IncH plasmids) would generate plasmid-free segregants 50% of the time, and would be expected to be rapidly lost from a growing population of cells. Nevertheless, many plasmids with low or very low copy number are completely stable, demonstrating loss rates of less than 0.01% per generation.

1. Multimer resolution

Many plasmids encode multimer resolution systems that resolve multimeric plasmid forms into discrete units. These systems serve to increase the number of independently segregating plasmids in the cell when it divides. The majority of multimer resolution systems characterized to date consist of a resolution site and a *trans*-acting resolvase/recombinase that acts to resolve multimers by recombination. The following table summarizes some of the known and suspected multimer resolution systems.

Table I-1 Multimer resolution systems

Plasmid	Site[a]	Protein[b]	References
F	*rsfF*	D protein	74, 106
P1	*loxP*	Cre	14
ColE1	*cer*	Xer	138, 140
RP4	*res*	ParA	37
pSC101	*psi*	Xer	28

[a]Site refers to the designation of the *cis*-acting site required for multimer resolution.

[b]Protein refers to the designation of the *trans*-acting protein required for multimer resolution.

2. Post-segregational killing

Another mechanism many plasmids employ in their fight for survival from one generation to the next is post-segregational killing of plasmid-free cells. In these systems, the plasmid encodes a poison capable of quickly killing the host cell or arresting its growth. The poison is prevented from acting by an antidote, which is also plasmid-encoded. The key to these systems is the comparative stability of poison and antidote. In each case, the poison has a much longer half-life than the antidote, such that should a plasmid-free segregant appear, the residual antidote in the cell will degenerate, allowing the poison to take effect, killing or arresting the cell in the process. The following table summarizes the most common killer systems.

Table I-2 Post-segregational killing systems

Plasmid	Poison[a]	Antidote[b]	References
R100/NR1/R1	PemK (protein)	PemI (protein)	157
R1	*hok* (RNA - encodes toxic protein)	*sok* (antisense to hok)	154, 155
F	*flmA* (RNA - related to hok)	*flmB* (RNA - related to sok)	77
F	CcdB (protein)	CcdA (protein)	143
P1	Doc (protein)	Phd (protein)	75
RK2	Psa (protein)	Psa (protein)	63

[a]Poison refers to the designation of the host lethal component

[b]Antidote refers to the designation of the antidote component required to override the lethal effects of the poison

3. Partitioning

Finally, many plasmids encode partitioning systems that actively partition plasmid copies to daughter cells during division. Although partition systems have been shown or suspected to exist in many plasmids of different incompatibility groups, the *sop* system of F and the *par* system of P1 are the best-known examples of these systems. Partitioning is thought to occur by pairing of plasmids with like partition systems. Paired plasmids are then separated at cell division by an as yet unknown mechanism, although it is thought that membrane and/or cytoskeletal interaction is involved in the

process (170). Active partitioning is critical to the stable maintenance of unit-copy plasmids such as P1, since at the time of division, only two copies of the plasmid exist in the cell.

Most well-known plasmid partitioning loci encode two proteins and a *cis*-acting site that are required for *par* activity. The *cis*-acting sites are thought to have centromere-like activity, allowing the plasmids to pair and segregate at cell division. In the *sop/par* systems encoded by F and P1, one protein (B) has been shown to bind to the *cis*-acting site (33), while the other (A) possesses ATPase activity (34) and may derive energy used in partitioning from hydrolysis of ATP. Computer simulations of plasmid R1 partition behaviour suggest that only one plasmid pair is partitioned in this system, with the remaining plasmid copies undergoing more or less random segregation (119). The following table lists some of the known plasmid partitioning systems.

Table I-3 Plasmid-encoded partition systems

Plasmid	Proteins[a]	Site[b]	References
F	SopA, SopB	*sopC*	97
P1	ParA, ParB	*parS*	4
RK2	ParB, ParC, ParD	*res*	117
NR1/R100	StbA, StbB	P_{AB}	142

[a]Proteins refers to the *trans*-acting factors required for active partitioning of the plasmid.

[b]Site refers to the *cis*-acting site required for active partitioning of the plasmid.

E. Conjugation

Many plasmids specify conjugation systems that enable their horizontal transmission from one host to another. In most cases, plasmid transfer requires the coordinated involvement of many gene products, and proceeds through several distinct phases. Since the majority of research in this area has concentrated on the F factor, this discussion will deal primarily with F as a paradigm for generalized gram negative plasmid transfer.

The first stage of conjugation involves initial contact between donor and recipient cells. This step is mediated by conjugative pili, which are expressed on the surface of the donor cell. The pilus contacts the surface of the recipient cell in an as yet unknown manner. Numerous structures have

been implicated as possible receptors for pilus attachment, including OmpA and LPS (5, 10), yet no one structure appears to be clearly required for this interaction. It remains a possibility that the interaction is non-specific or that multiple receptors and/or mechanisms may enable mating pair formation.

Once the pilus has made contact with an appropriate recipient cell, the two cells form a close pair, which in initial stages is unstable and subject to dissociation. Later, the mating pair is stabilized by the action of certain plasmid-encoded gene products, becoming less susceptible to dissociation. At this point some sort of pore or channel exists as a conduit between the donor and recipient cells. It is not clear as to the composition or form of this pore, but Harrington and Rogerson have shown that conjugation can occur without direct cell-cell contact (51), suggesting that the pilus itself can act as a channel for plasmid DNA transmission.

A mating signal, the exact nature of which is currently unknown, triggers the actual transfer of the donor plasmid. A number of plasmid-encoded proteins are involved in a process whereby the donor plasmid is nicked at a specific site (known as oriT). The nicked DNA strand is then transferred in a 5'→ 3' orientation from the donor cell, across the mating bridge, and into the recipient cell. In some instances, donor proteins are transferred as well, possibly by covalent attachment to the 5' end of the transferred DNA strand (115). The transfer is typically a replicative event

where the transferred donor DNA strand is replaced by the action of DNA polymerase III. However, replication is not required for transfer of DNA to the recipient cell (168, 169).

Once a complete copy of the donor plasmid is transferred to the recipient cell, transfer is terminated. The single strand transferred to the recipient is discontinuously replicated during transfer (utilizing a plasmid-encoded or host-encoded primase) to provide an intact double-stranded copy of the original plasmid. The donor and recipient cells then dissociate. The entire process requires several minutes to complete.

Recently, plasmid transfer systems have been shown to be highly promiscuous, capable of transferring DNA between very distantly related organisms, and even in some cases between species of different kingdoms (54, 137). Even transfer systems encoded by so-called "narrow host range" plasmids such as F, are capable of inter-kingdom transfer (54). This suggests that the major limiting factor for host range is not transfer, but establishment and maintenance of the plasmid.

1. The F Tra Operon

The F plasmid and its many close relatives possess some 38 genes and open reading frames that have been implicated in the transfer process. Transfer genes have been designated *tra* or *trb* (the *trb* loci were designated after *traA* through *traZ* were assigned) and encompass approximately 33 kb

of the plasmid (41). The majority of these lie in a single large operon. Numerous functional studies have shown the *tra/trb* genes to be involved in distinct processes of conjugation. The following table summarizes these data:

Table I-4 Transfer genes encoded by the F plasmid

Transfer Process	Genes Involved	Reference(s)
Pilus Synthesis	*tra*A, B, C, E, F, G, H, K, L, Q, U, V, W, X	43, 93, 126, 72, 172, 39, 49, 173, 95, 86, 30, 85
	*trb*C	
Mating Pair Stabilization	*tra*G, N	39, 84
Surface Exclusion	*tra*S, T	61, 92
DNA Metabolism	*tra*D, I, M, Y	60, 90, 36, 99
Regulation	*tra*J	31, 176, 159
	*fin*O, P	

2. Other Transfer Systems

Although the F plasmid is the most extensively studied with respect to its conjugation system, several other plasmid transfer systems have been studied in detail in recent years. These include the IncP plasmids (RP4/RK2), the IncW plasmids (R388), the IncQ plasmids (RSF1010), and the Ti plasmids of *Agrobacterium tumefaciens*. Although the transfer genes present on these plasmids and their organization in most cases differ

considerably from F, a number of homologous functions exist. Particularly in the case of the genes directly involved in the processes of signalling, origin nicking, and DNA transport, functional and/or sequence similarity is observable in all of these plasmid groups. The following table summarizes these similarities:

Table I-5 Functional homologies between plasmid transfer proteins involved in DNA metabolism during conjugation

	Related Proteins[a]			
F Protein	RP4/RK2	R388	RSF1010	Ti
TraI	TraI	TrwC	MobA	VirD2
TraY	TraJ	TrwA	MobC	
TraM	TraK		MobB	
TraD	TraG	TrwB		VirD4

[a]Tra protein comparisons are based on sequence homology, size similarity, and/or functional similarity (41, 64, 76, 109).

3. Conjugative Pili

The conjugative pilus is the primary mediator of conjugal DNA transfer in many gram negative systems. It is involved in the initial steps of mating pair formation and without a functional pilus, transfer cannot occur. Conjugative pili have been characterized morphologically into three basic classes: thick, flexible pili (8-11 nm in diameter), thin, flexible pili (~6 nm in

diameter), and short, rigid pili (22). Plasmids that express short, rigid pili generally demonstrate surface-preferred or surface-obligatory mating systems, and transfer very poorly in liquid culture. The presence of flexible pili provides for conjugative transfer that is equally efficient on a solid surface as it is in a liquid medium. This is likely due to the ability of longer, flexible pili to initiate and establish contact with potential recipient cells at much higher frequency than short rigid pili under conditions where donor and recipient cells are dispersed. Broad-host range plasmids of the N, P, and W incompatibility groups all produce short rigid pili, and therefore exhibit strong surface preference for transfer.

In terms of biochemical and molecular characterization of conjugative pili, those of the F plasmid and its close relatives have been studied most extensively. The F pilus is characterized morphologically as a thick flexible pilus (8 nm diameter) and can attain lengths of up to 20 μm. The F-pilus is composed of a single protein subunit (pilin), the product of the *traA* gene. The pilin monomer is arranged in a helical array with five subunits per turn (110). When viewing isolated pili filaments by electron microscopy, the proximal end of the F pilus often has a vesicle-like knob which consists of disarrayed pilin subunits (42). The distal end of the pilus appears to be tapered, and may contain a unique arrangement of pilin monomers, or a minor pilin protein that lends itself to recipient recognition and contact.

As mentioned, the pilin monomer is encoded by the *traA* gene. This

gene encodes a 121 amino acid protein that is processed to a mature pilin of 70 amino acid residues. The product of the *traQ* gene is necessary for proper maturation of the pilin subunits. The TraQ protein may protect the TraA protein from degradation and act as a chaperone for the insertion of propilin in the cytoplasmic membrane for proper processing (83). A large proportion of the mature pilin subunits are found to be acetylated at their amino terminus; the product of the *traX* gene is required for this acetylation to occur (94). F pilin monomers pool in the inner membrane, from which they are assembled into F pili (96).

At least 12 other gene products are involved in or affect pilus assembly (see Table I-4). The precise role of these proteins is currently unknown. Inactivation of most pilus-assembly gene products prevents the expression of detectable F pili, but has no effect on production of mature pilin monomer. Recently, as more sequence data has become available for other plasmid systems, weak homologies have emerged with certain F-pilus assembly genes (64, 41, 112). These are outlined in Table I-6. Of note is the homology detected between TraA, B, C, and L and proteins implicated in pertussis toxin secretion (129).

Table I-6 Proteins with homology to F pilus assembly gene products

F Pilus Assembly Gene Product	Homologous Proteins
TraA	VirB2 (Ti), TrwM (IncW), TrbC (IncP), TraM (IncN), PtlA (*B. pertussis*)
TraC	VirB4 (Ti), TrwK (IncW), TrbE (IncP), TraB (IncN), PtlC (*B. pertussis*)
TraL	VirB3 (Ti), TrwL (IncW), TrbD (IncP), TraA (IncN), PtlB (*B. pertussis*)
TraB	VirB10 (Ti), TrwE (IncW), TrbI (IncP), TraF (IncN), PtlG (*B. pertussis*)
TraK	TrbG (IncP)
TraV	TrbH? (IncP)

F. IncH Plasmids

Plasmids of the H incompatibility type originally gained notoriety in the early 1970's when they were discovered to be the source of chloramphenicol resistance in several large outbreaks of typhoid fever in Mexico, India, Vietnam, and Thailand (8, 9). Most of the H plasmids carried by the *Salmonella typhi* strains involved in these outbreaks also mediated resistance to streptomycin, sulphonamides, and tetracycline. Later, resistance to ampicillin, kanamycin, and trimethoprim was also discovered to

be associated with the carriage of H plasmids in various other *Salmonella* strains (8).

1. Characteristics of H plasmids

Several features are characteristic of H plasmids. They are among the largest Enterobacterial plasmids isolated, with sizes ranging from 150-kb to greater than 250-kb. Their host range includes most species of the *Enterobacteriaeceae*, as well as certain other gram negative bacteria (80). All H plasmids (with the exception of the sole member of the IncHI3 group, MIP233) produce antigenically and morphologically related conjugative pili that are classified as thick and flexible (22). Most H plasmids carry antibiotic resistance genes, with some carrying resistance to more than seven different antibiotics. In addition, genes encoding mercury resistance, tellurite resistance, phage inhibition, colicin resistance, and citrate utilization have been discovered in various H plasmids.

2. Classification of H plasmids

The IncH complex comprises a large group of related plasmids. This complex consists of two incompatibility groups: IncHI and IncHII. In addition, the IncHI group has been subdivided into three subgroups (IncHI1,

IncHI2, and IncHI3) on the basis of DNA-DNA hybridization and incompatibility studies (120, 167).

The IncHI Group

The IncHI group was originally divided into two subgroups, on the basis of one-way incompatibility with the F factor (134). Plasmids of the HI1 (originally H1) group display vectorial incompatibility when introduced into a host cell along with the F factor. In contrast to this, plasmids of the IncHI2 (originally H2) group are compatible with F. Furthermore, representative plasmids of each group possess relatively little homologous DNA (167). Although plasmids of each HI subgroup are incompatible with one another, the incompatibility observed between plasmids of different HI groups is considerably weaker than that observed between plasmids of the same HI subgroup (151).

Plasmids of the IncHI2 subgroup can be further distinguished from those of the IncHI1 group by a number of characteristics. Many plasmids of this group specify resistance to tellurite (62), phage inhibition of a number of different bacteriophages (148), and production of colicin B (118). None of these traits are typically carried by plasmids of the IncHI1 subgroup.

Plasmid MIP233, the sole member of the HI3 subgroup, was classified as an H plasmid on the basis of incompatibility with HI1 and HI2 plasmids, although DNA-DNA membrane filter hybridization studies revealed the

presence of little homology with these groups (120, 167). Furthermore, MIP233 produces serologically and morphologically distinct conjugative pili that are unrelated to those encoded by other H plasmids (21).

The IncHII Group

Plasmids of the IncHII group specify H pili, and are characteristically large, yet they are compatible with IncHI plasmids, hence the classification as a second H incompatibility group. The few members of this incompatibility group characterized to date have been isolated from strains of *Klebsiella aerogenes* (32). Like plasmids of the IncHI2 group, IncHII plasmids specify phage inhibition of λ, T1, T5, and T7 phages (148, 23), as well as resistance to tellurite and colicin B (79).

3. Conjugation

All H plasmids encode conjugation systems. The IncHI plasmids are unique in that they specify a temperature-sensitive transfer mechanism (147). Transfer of these plasmids to recipient cells occurs most efficiently at temperatures of 26°C - 30°C, whereas transfer at 37°C is greatly reduced, often to the point that it is unobservable. This is in contrast to the great majority of conjugative plasmids whose transfer efficiency peaks at temperatures near 37°C, and is drastically reduced at lower temperatures. It

has been suggested that this unique mode of transfer may be an important mechanism in the dissemination of plasmid-encoded genes in soil or aquatic environments (135).

IncHII plasmids, by contrast, exhibit transfer frequencies that are comparable at 26°C and 37°C. Furthermore, whereas most IncHI plasmids possess repressed conjugation systems and transfer at relatively low frequencies, the IncHII plasmids examined appear to be relatively transfer-derepressed (175). In conjugations of equal duration, IncHII plasmids tend to transfer at rates approximately 100-fold greater than those observed for wild-type IncHI plasmids.

4. Molecular and genetic analysis of H plasmids

Representative members of incompatibility groups HI1, HI2, and HII have been studied in some detail. Restriction endonuclease maps have been generated and cloning and mutational analysis has defined coding regions for numerous genetic elements present within these plasmids.

R27 (IncHI1)

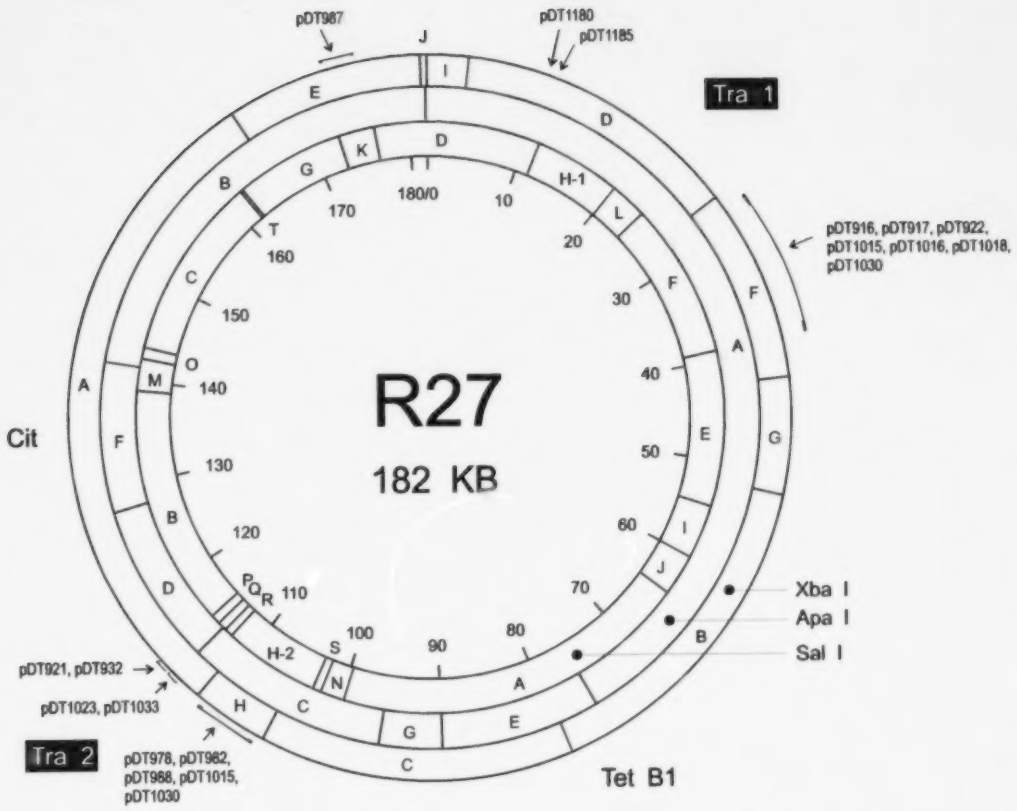
R27 is the best-studied H plasmid of the IncHI1 subgroup. The plasmid was originally isolated from a strain of *Salmonella typhimurium* in 1961. This 182-kb plasmid specifies resistance to tetracycline (144, 149), and

encodes genes for the utilization of citrate as a sole carbon source (150). A restriction map of R27 has enabled the physical location of these and other traits on the plasmid (150, 152, see Figure I-1). A small region of the plasmid was found to be responsible for the observed one-way incompatibility with the F-factor that all HI1 plasmids exhibit (153, 123). Sequence analysis of this region revealed the presence of a partial replicon with strong homology to RepFIA, a replicon present in many F plasmids. Transposon insertion mutagenesis has defined two broad regions of the plasmid that are involved in the production of a functioning transfer apparatus (144, 152).

Most of the genome of R27 has been cloned into cosmid vectors (100). Fragments encompassing all but a small region (~8-kb in size) are present in this cosmid library, as depicted in Figure I-2. The unrepresented region (a portion of the S*al*I-H2 fragment) could not be cloned, despite numerous attempts using a variety of cloning vehicles. This library proved to be very useful for a number of experiments presented in this thesis.

Figure I-1 Restriction map of R27

Restriction endonuclease cleavage sites and fragments are indicated in the outer circles. These sites are oriented with respect to the *Xba*I site at coordinate 0 of the map numbered (in kilobases) from that site. The restriction fragments are labelled alphabetically in decreasing order of size. Numbers near the outer circle represent Tn5 or Tn7 insertion sites resulting in a transfer-deficient phenotype; the position corresponds to the insertion site of the transposon. The two main transfer regions are designated Tra1 and Tra2. Other designations: Cit, citrate utilization; Tet B1, tetracycline resistance determinant B1.


R27
182 KB
pDT987
pDT1180
pDT1185
Tra 1
pDT916, pDT917, pDT922,
pDT1015, pDT1016, pDT1018,
pDT1030
Cit
Xba I
Apa I
Sal I
pDT921, pDT932
pDT1023, pDT1033
Tra 2
pDT978, pDT982,
pDT988, pDT1015,
pDT1030
Tet B1
180/0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170

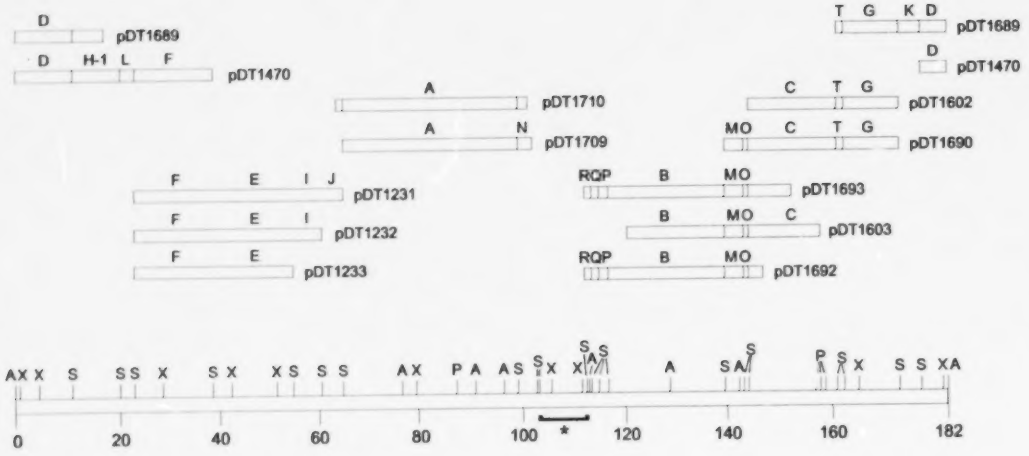


Figure I-2 Cosmid subclones of R27

Linear restriction map of R27 (182 kb), showing regions cloned into cosmids. Fragments are lettered according to the nomenclature shown in Figure I-1. The *Xba*I (X), *Sal*I (S), and *Apa*I (A) restriction sites are indicated in the map. The unclonable region is indicated by an *.

R478 (IncHI2)

R478, a member of the IncHI2 incompatibility group, specifies resistance to Tc, Cm, Km, Te, Hg, arsenite, and arsenate. It was first isolated from an strain of *Serratia marcescens* in 1969 (53). Recently, a restriction map of the plasmid was constructed using the enzymes X*ho*I, X*ba*I, A*pa*I, and S*al*I (165). Restriction mapping and subcloning demonstrated the plasmid to be approximately 272-kb in size. Determinants encoding resistance to colicin B, Te, Hg, arsenite/arsenate, Cm, Km, and Tc have been located on the restriction map of R478 (165).

pHH1508a (IncHII)

This plasmid was originally isolated from a clinical isolate of *Klebsiella aerogenes* (32). It carries resistance to tellurite, streptomycin, spectinomycin, and trimethoprim (23, 32, 175). A restriction endonuclease map of the plasmid has been constructed and used to accurately size the plasmid at 208-kb. Transposon insertion mutagenesis was used to generate transfer-deficient mutants of the plasmid. Mapping of the transposon insertions that produced a Tra$^-$ phenotype revealed *tra* genes to be widely scattered throughout the plasmid (175).

G. Objectives of this study

In this study, molecular analysis of the maintenance and transfer determinants of R27 was undertaken. Two replicons and an incompatibility determinant were identified and mapped on the plasmid. One replicon was cloned and sequenced and its replicative properties were characterized. *Cis* and *trans* acting components of the replicon were identified and comparisons made with replicons of other plasmids. The effect of these maintenance elements on plasmid stability was investigated.

The nature of the temperature sensitivity exhibited by the transfer mechanism of IncHI plasmids was investigated and transposon insertion mutagenesis of R27 was undertaken in an effort to characterize regions of the plasmids involved in transfer, and more specifically, pilus synthesis. Conjugative pili produced by IncHI plasmids were isolated and characterized, allowing comparison with other conjugative pili. Nucleotide sequence analysis of regions of the plasmid involved in pilus synthesis was performed.

Close scrutiny of the mechanisms this plasmid employs in its horizontal and vertical transmission will assist in understanding the function of these systems. This work will also help to understand the evolutionary relationships that exist between plasmids of diverse origin that occupy various ecological niches.

Chapter II Materials and Methods

1. Antibiotics and media

Growth media used included the following: Luria Broth (Difco), Brain Heart Infusion (Oxoid), MacConkey (Difco), and Diagnostic Sensitivity Testing Agar (Oxoid). If necessary, agar was added to a final concentration of 1.5% (w/v) for solid media. Unless otherwise stated, antibiotics were used in the concentrations specified in Table II-1.

Table II-1 Antibiotics used in this study

Antibiotic (Abbreviation)	Concentration (μg/ml)
Ampicillin (Ap)	100
Carbenicillin (Cb)	500
Chloramphenicol (Cm)	25
Kanamycin (Km)	30
Nalidixic Acid (Nal)	40
Potassium Tellurite (Te)	50
Rifampicin (Rif)	100
Streptomycin (Sm)	200
Tetracycline (Tc)	10
Trimethoprim (Tp)	10

2. Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table II-2 and Table II-3. These strains and others created in the study were stored at -80°C in a sterile preservative medium (BHI broth, 20% glycerol).

Table II-2 Bacterial strains used in this study.

E. coli strain	Relevant Characteristics	Reference or Source
HB101	pro leu thi lacY hsdR end recA rpsL20 ara-14 galK2 xyl-5 mtl-1 supE44 Str^R	19
DH5α	F^- supE44 Δ(lacZYA-argF)U169 (ϕ80 lacZΔM15) recA1 endA1 gyrA96 thi-1 relA1 hsdR17(r_K^-,m_K^+) λ^- deoR	50
JE2571-1	leu thr fla pil str Rif^R	20
JE2571-2	leu thr fla pil str Nal^R	20
J53-1	pro met Nal^R	15
JC1569	F^- argG6 his-1 metB1 leu-2 lacY1 gal-6 xyl-7 recA1 Nal^R	15
DH10B	F^- mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80dlacZΔM15 ΔlacX74 deoR recA1 endA1 araD139 Δ(ara,leu)7697 galU galK λ^- rpsL hupG	GibcoBRL, Burlington, Ontario
SF800	thy polA Nal^R	35
RG192	ara leu lac^- Rif^R	144
JF1125	rk^- mk^- recBC lop11 leuB thr thiA	R. W. Davis

Table II-3 Plasmids used in this study.

Plasmid	Resistance Markers	Use	Reference or Source
R27	Tc	IncHI1 prototype	147
pDT1942	Tc, Km	derepressed Tra mutant of R27	81
pUC13	Ap	cloning vector	160
pUC118	Ap	cloning vector	161
pUC21	Ap	cloning vector	162
pUCBM20	Ap	cloning vector	Boehringer Mannheim
pMS119	Ap	expression vector	139
pKIXX	Km	Km^R cassette	Pharmacia
pULB2436	Ap	IncHI1 *inc* probe	29
pHH1508a	Te Tp Sm	IncHII prototype	23
pJL207	Cm	promoter probe vector	7, 25
pNK2884	Cm	mini-Tn10 transposon delivery plasmid	68

3. Conjugation

Conjugation experiments were performed in liquid culture as previously described (147). Briefly, isolated colonies of both the donor and recipient strains were inoculated to broth media (Penassay or BHI) and incubated overnight at either 28°C or 37°C. A 1:500 dilution of the overnight

culture was made with fresh media and incubation continued for 4 - 6 hours. 10 µl of the donor culture and 50 µl of the recipient culture were then mixed in 1.5 ml of fresh media and incubated for an additional 1 - 18 hours. The mating mixture was then diluted in phosphate buffer and various dilutions were plated on selective media to determine transfer frequency (expressed as transconjugants per donor or transconjugants per recipient).

4. Mini plasmid prep

Small-scale plasmid DNA preparations (1-20 µg) were prepared using a modification of the alkaline-SDS method described by Birnboim and Doly (17). Overnight cultures (1.5 ml) of an appropriate *E. coli* strain harboring the plasmid of interest were transferred to 1.5 ml microfuge tubes and subjected to brief centrifugation at maximum speed to sediment the cells. After decanting the supernatant, the cells were resuspended in 100 µl of solution 1 (40 mM Tris, pH 8.0, 2.5 mM EDTA, 10% glucose). 200 µl of solution 2 (0.2N NaOH, 1% SDS) was then added and the sample mixed by gentle inversion until the solution cleared. The sample was mixed with 150 µl of solution 3 (3M sodium acetate, pH 5.2), and the resulting precipitate was removed by centrifugation at maximum speed for 5 min. The supernatant was mixed with 900 µl of ethanol, and incubated at -80°C for 15 min. Nucleic acids were collected from the sample by centrifugation at maximum speed for 5 min. The resulting pellet was dried briefly under

vacuum and resuspended in 100 µl TE buffer (10 mM Tris, pH 7.5, 1 mM EDTA) containing 20 µg/ml of Dnase-free pancreatic Rnase.

Where experimental procedures required, plasmid DNA isolated by this procedure was further purified by extraction with phenol:chloroform, or by using the Magic Minipreps plasmid DNA purification system (Promega, Madison, WI) according to the manufacturer's directions.

5. Large scale plasmid prep

Large scale preparations of plasmid DNA (10 - 500 µg) were prepared using a scaled up version (100-fold) of the mini-method described above. Final purification of plasmid DNA was performed by cesium chloride ethidium bromide buoyant density gradient ultracentrifugation, as follows. Crude DNA preparations were dissolved in 9 ml of TE buffer (1mM EDTA, 10 mM Tris, pH 7.5), and 9 g of solid cesium chloride was added to the solution. After transferring to an appropriate sealable ultracentrifuge tube, 150 µl of an ethidium bromide solution (10 mg/ml) was added to the solution. After sealing, the tubes were subjected to centrifugation in a Beckman L-80 ultracentrifuge for 20 - 24 hours at 55,000 RPM (Ti70 rotor). Alternatively, samples were subjected to centrifugation for 4 - 6 hours at 65,000 RPM in an NVT65 rotor.

After centrifugation, plasmid DNA was removed from the centrifuge tube under UV illumination, extracted several times with isoamyl alcohol to

remove ethidium bromide, diluted three-fold with TE buffer, and mixed with two volumes of 95% ethanol. The plasmid DNA was recovered by centrifugation, after incubation at -20°C for 30 min. (Beckman JA-20 rotor, 10,000 RPM). The purified plasmid DNA was resuspended in 0.1 - 1.5 ml TE buffer and stored at 4°C.

6. Isolation of total DNA

Total cellular DNA was isolated from 1 ml of bacterial culture by the following method. Cells were subjected to centrifugation in an Eppendorf microfuge at maximum speed for 5 minutes. The cell pellet was resuspended in 150 µl of buffer (50 mM Tris, pH 8.0, 10 mM EDTA, 25% sucrose, 5 mg/ml lysozyme), and incubated at 37°C for 30 minutes. The mixture was extracted with phenol-chloroform, and nucleic acids were precipitated from the aqueous phase with 300 µl of 95% ethanol after the addition of 15 µl of sodium acetate (3M, pH 5.2). After centrifugation, the DNA pellet was dried, dissolved in TE buffer, and stored at 4°C for further use.

7. Transformation

Plasmid DNA and ligation mixtures were used in the transformation of competent *E. coli* cells by the method of Cohen *et al.* (27). In this method, 0.5 ml of an overnight culture of the desired *E. coli* strain was inoculated to 25 ml of BHI broth. The culture was incubated at 37°C with vigorous agitation for 2 hours. Cells were collected by centrifugation (1 min., 7000 RPM,

Beckman JA-20 rotor) and resuspended in 15 ml of ice cold calcium chloride solution (50 mM $CaCl_2$, 10 mM Tris, pH 7.5). After resting on ice for 30 min., the suspension was subjected to centrifugation (30 seconds, 7000 RPM, Beckman JA-20 rotor) and the cell pellet gently resuspended in 1 - 2 ml of ice cold calcium chloride solution.

DNA to be used in the transformation (0.1 - 2.0 μg) was added to 100 μl of the cell suspension and incubated on ice for at least 20 min. The mixture was then subjected to a 42°C heat shock (by immersion in a water bath) for 2 min. After returning the cells to ice, 200 μl of LB broth was added, and the mixture placed in a 37°C water bath for 20 - 30 min. Aliquots of the transformed cells were then plated on selective media.

8. Agarose gel electrophoresis

Agarose gel electrophoresis of nucleic acids was performed using standard agarose (Promega, Madison, WI) or low melting point agarose (Promega, Madison, WI) at concentrations ranging from 0.5% - 2% (w/v), depending on the size of the DNA to be separated. Buffers employed in the electrophoresis were either TBE (0.09 M Tris, 0.09 M boric acid, 2.0 mM EDTA, pH 8.3) or TAE (0.04 M Tris, 0.04 M acetic acid, 1.0 mM EDTA, pH8.3). Electrophoresis was typically carried out at 5 - 10 V/cm for various time periods. After electrophoresis, gels were stained by submersing in a

dilute solution of ethidium bromide (5 μg/ml) for 20 - 30 min. DNA was visualized under illumination with UV light (260 nm).

9. Restriction digestion

Restriction endonucleases were obtained from several sources (Boehringer Mannheim Biochemicals, Canada, Ltd.; Bethesda Research Laboratories Canada, Ltd.; or New England BioLabs, Ltd.), and digestions carried out according to manufacturers recommendations using the supplied buffers. Where possible, double or triple digests were performed simultaneously, using a mutually compatible buffer, or separately with ethanol precipitation between digestion with each enzyme. Digested DNA that was to be analyzed by agarose gel electrophoresis was mixed with 1/10 volume of bromophenol blue loading dye (48% sucrose, 0.25% bromophenol blue, 12 mM EDTA).

Digested DNA that was to be used in further manipulations was desalted as follows. The sample was diluted eight- to ten-fold in diluent (100 mM sodium acetate, 10 mM Tris, pH 7.5). Two volumes of ethanol were added, the sample mixed, and placed at -80°C for 15 min. Precipitated DNA was collected by centrifugation (14,000x*g*, 5 min.), washed briefly in 70% ethanol, dried under vacuum, and resuspended in an appropriate buffer.

10. Purification of DNA restriction digest fragments

In cases where a particular DNA fragment from a restriction digest was to be used in other experiments, the fragment was separated and purified from low-melting-point agarose. After subjecting DNA fragments to electrophoresis, the gels were stained as described and fragments were excised from the gel quickly, so as to minimize exposure to the UV light used to visualize the DNA. The excised fragments were placed in 100 µl of TE buffer and heated to 65°C for 10 min. to melt the agarose. An equal volume of phenol (Tris-equilibrated) was added, the solution mixed vigorously, and phases separated by centrifugation in a microfuge for 3 min. The aqueous phase was carefully removed, and extracted with an equal volume of butanol, followed by extraction with an equal volume of chloroform. DNA in the aqueous phase was precipitated by the addition of 1/10 volume of sodium acetate (3M, pH 5.2) and 2 volumes of ethanol.

11. Ligation of DNA fragments

DNA restriction fragments with appropriate compatible ends were used in ligations as follows. Purified DNA fragments to be ligated were mixed in a small volume (<25 µl) with ligation buffer (66 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM DTE, 1 mM ATP, pH7.5) and 1-2 units of T4 DNA ligase. The ligation was allowed to proceed for 2 - 18 hours at 16°C, or in some cases, for

24 hours at 4°C. Small volumes of the ligation mixture were used to transform competent cells, as described.

12. Exonuclease III digestion of DNA

Exonuclease III was used to produce deletion derivatives of cloned DNA fragments. Appropriate restriction endonucleases were chosen to generate two cuts in the target plasmid, one of which was susceptible to exonuclease III. Approximately 5 - 10 μg of DNA was used in each reaction. After digestion, the DNA was precipitated and desalted by the addition of eight volumes of diluent (100 mM sodium acetate, 10 mM Tris, pH 7.5) and ethanol. The DNA was resuspended in 120 μl of exonuclease buffer (50 mM Tris, pH 7.2, 6.5 mM $MgCl_2$) and equilibrated at 37°C. Exonuclease III (500 U) was added to the solution, and 5 μl samples were removed at 30 sec. intervals. Each sample was transferred to 15 μl of ice cold S1 reaction mixture (0.25 M NaCl, 0.03 M potassium acetate, pH 4.5, 5% glycerol, 0.2 mM $ZnSO_4$, S1 nuclease, 200 U/ml).

After all samples were taken, the mixtures were incubated at 30°C for 30 min and 2 μl of stop solution (0.3 M Tris, 50 mM EDTA, pH 8.0) was added to each. Samples were incubated at 65°C for 10 minutes and then pooled. The nucleic acids in each pool were precipitated with the addition of 1/10 volume of sodium acetate (3M, pH 5.2) and two volumes of ethanol. The precipitated DNA was collected by centrifugation, washed in 70% ethanol,

and resuspended in ligation mixture (66 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM DTE, 1 mM ATP, pH 7.5, T4 DNA ligase, 10 U/ml). Ligation was allowed to proceed overnight at 4°C or 16°C and ligation products were used in the transformation of chemically competent *E. coli*.

13. Nick Translation

DNA restriction fragments were labelled with [α-^{32}P]dCTP by nick translation according to the method of Rigby *et al.* (116). Purified DNA was mixed in the labelling buffer (dATP, 20 μM, dGTP, 20 μM, dTTP, 20 μM, $MgCl_2$, 2.5 mM, β-mercaptoethanol, 5 μM) and 50 μCi of [α-^{32}P]dCTP was added. After the addition of 2 x 10^{-7} μg of DNAse, the mixture was incubated at 15°C for 15 minutes. 0.5 units of *E. coli* DNA Polymerase I was added to the solution, and incubation continued for an additional 2 - 4 hours. Labelled DNA was recovered from solution by ethanol precipitation after the addition of 1/10 volume of sodium acetate (3M, pH 5.2) and 10 - 20 μg of yeast tRNA. After washing in 70% ethanol, the DNA pellet was resuspended in TE buffer and a small quantity analyzed in a Beckman LS6800 liquid scintillation counter to determine specific activity.

14. Hybridization of nucleic acids

DNA separated in agarose gels was transferred to nitrocellulose (Bio-Rad Laboratories, Hercules, CA) or nylon membranes (Micron Separations Inc., Westborough, MA) by the method of Southern (136).

Colony blots were prepared as described (88) by lifting bacterial colonies from a plate with a nitrocellulose membrane. The membrane was then placed on a piece of Whatman 3MM filter paper that had been previously soaked in 10% SDS. After 5 min., the membrane was transferred to a piece of filter paper soaked in denaturing solution (1.5 M NaCl, 0.5 M NaOH) and incubated for an additional 5 min. After transferring to a piece of filter paper soaked in neutralizing solution (2 M NaCl, 1 M Tris, pH 5.5) for 5 minutes, the membrane was finally rinsed briefly in 2X SSC and air dried.

Dot blots were prepared as described previously (65) by spotting DNA (denatured in 0.3 M NaOH at room temperature for 15 min.) onto nitrocellulose or nylon membranes (soaked in 1 M ammonium acetate) using a dot blot apparatus. The filter was then air dried.

Hybridization of nucleic acids was carried out according to the method of Portnoy *et al.* (113). Nitrocellulose or nylon filters were incubated in hybridization solution (50% formamide, 2X SSC, 0.1% SDS, 1 mM EDTA, 0.2% ficoll 400, 0.2% BSA, 0.2% polyvinyl pyrrolidone, 50 μg/ml sonicated herring sperm DNA) for 2 - 4 hours at 37°C or 42°C. The radiolabelled DNA probe (1.0 - 5.0 x 10^6 CPM) was denatured by boiling for 10 min. with 500 - 750 μg of sonicated herring sperm DNA. The denatured probe was added to the hybridization solution, and incubation continued for an additional 16 - 18 hours with gentle rocking motion. The filter was then transferred to wash solution (2X SSC, 0.1% SDS, 1 mM EDTA, pH 7.0) and incubated with gentle

agitation at room temperature for 5 mins. After replacing the wash buffer, incubation was continued at 65°C for an additional 45 min. This step was repeated once. Finally, the blot was briefly rinsed in 1X SSC and air dried (or wrapped in Saran wrap). The filter was then exposed to Kodak X-ray film (X-Omat AR) at -80°C for various time periods.

15. Isolation of total cellular RNA

Total cellular RNA was isolated from exponentially growing *E. coli* cultures by the method of Aiba *et al.* (6). Cells were collected from a one ml culture by centrifugation in a microfuge and resuspended in 300 µl of RNA buffer (10 mM Tris, 100 mM NaOAc, 5 mM $MgCl_2$, pH 5.3). After placing the mixture in a 65°C water bath, 30 µl of 10% SDS was added and the solution mixed by inversion. An equal volume of phenol (equilibrated with RNA buffer and prewarmed to 65°C) was immediately added and the mixture shaken. Chloroform (300 µl) was added to the solution, mixed, and centrifuged at maximum speed in a microfuge for 2 - 3 min. The aqueous phase was removed and re-extracted two or three times with phenol/chloroform, followed by precipitation with 1M ammonium acetate and ethanol. The nucleic acid pellet was dried and stored at -20°C or resuspended in 150 µl of water and used immediately. DNA was removed by the addition of RNase-free DNase (final concentration, 1 µg/ml), and the concentration of RNA estimated by measuring absorbance at 260nm (1 A_{260} unit = 40 µg/ml).

16. Primer extension

Primer extension was carried out as follows. Approximately 6 μg RNA and 20 ng of the primer were heated at 65°C in 10 ml of AMV reverse transcriptase buffer (50 mM Tris, 50 mM KCl, 7 mM $MgCl_2$, pH 8.3) for 2 minutes, followed by gradual cooling to 30°C over a 20 minute period. AMV reverse transcriptase was then added (3 units), along with [α-^{35}S]dATP (30 mCi), dCTP, dGTP, dTTP (0.1 mM each) and DTT (5 mM). The mixture was incubated at 42°C for 2 minutes. After adjusting the concentration of dATP to 0.1 mM, the reaction was continued for 20 minutes, at which point 20 μl of sequencing stop solution was added. The samples were then heated at 85°C for 3 minutes, and loaded onto a 7% polyacrylamide sequencing gel.

17. Polymerase chain reaction

DNA fragments were amplified by the polymerase chain reaction according to the following method (121). Template DNA (0.1 μg) was mixed with 100 pmoles of each primer in 100 μl of amplification buffer (50 mM KCl, 100 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.01% gelatin). 2.5 units of Taq DNA polymerase was added to the mixture, and a drop of light mineral oil was overlaid. The samples were transferred to a Perkin-Elmer 9600 thermocycler, and amplification was performed for 25-35 cycles. Amplification parameters were as follows: 94°C, 1 min.; 50°C, 2 min.; 72°C, 2-3 min.

18. Preparation of cellular protein for SDS-PAGE

Total bacterial protein was prepared by the following method. A one ml sample of a logarithmic phase culture was withdrawn, and cells recovered by brief centrifugation. 100 µl of SDS-PAGE solubilization buffer (62.5 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol (v/v), 0.00625% bromophenol blue) was added and the cells resuspended with a pipette tip. The sample was heated to 97°C for 3-4 min., cooled on ice, and insoluble material removed from the solution by centrifugation (14,000x*g*, 5 min.). A small sample (2-20 µl) was loaded onto an SDS polyacrylamide gel and subjected to electrophoresis as described below.

19. Analysis of proteins by polyacrylamide gel electrophoresis

Polyacrylamide gels (10 - 16%) containing 1% SDS were prepared according to the method of Laemmli (71). After electrophoresis, gels were fixed and stained in Coomassie Blue staining solution (40% methanol, 10% acetic acid, 0.2% Coomassie Brilliant Blue R250). Gels were then destained by washing in destaining solution (30% methanol, 10% acetic acid) for several hours.

Silver staining of polypeptides separated by SDS-PAGE was performed as described by Sambrook *et al.* (121). After fixation, gels were washed twice in 30% ethanol with gentle agitation fro 30 min. Following this, three washes in Milli-Q water were carried out for 10 min. each with gentle

agitation. The gels were then submersed in a 0.1% (w/v) solution of $AgNO_3$ and incubated for 30 min. After a thorough rinsing with Milli-Q water, gels were immersed in developing solution (2.5% Na_2CO_3, 0.02% formaldehyde) for several minutes. Development was stopped by the addition of 1% acetic acid.

20. High resolution SDS polyacrylamide gel electrophoresis

In order to resolve small polypeptides on polyacrylamide gels, the method described by Schägger and von Jagow was used (125). Briefly, gels were cast with three separate polyacrylamide layers: a 16.5% separating gel, a 10% spacer gel, and a 5% stacking gel. All gel layers contained the same buffer (1 M Tris, pH 8.45, 0.1% SDS). Separate cathode (0.1 M Tris, 0.1 M Tricine, 0.1% SDS, pH 8.25) and anode (0.2 M Tris, pH 8.9) running buffers were used during the electrophoresis. After electrophoresis, polyacrylamide gels were stained with Coomassie Blue or silver as described above.

21. In vitro transcription-translation

In vitro transcription-translation analysis was accomplished with the use of a procaryotic DNA-directed translation kit (Amersham Canada Limited, Oakville, Ontario), and the experiment was carried out according to the manufacturer's instructions. Translation products were labelled with L - [^{35}S] methionine and subjected to electrophoresis in SDS-polyacrylamide gels. After fixing with 7% acetic acid, the gels were soaked in 1M sodium salicylate

(pH 6.0) for 30 minutes, dried, and exposed to X-Ray film at -70°C for various time periods.

22. β-Galactosidase activity assay

β-Galactosidase activity was assayed by the method of Miller (91). *E. coli* cultures were incubated overnight in A medium and the appropriate antibiotic. After dilution (1:50) in fresh media, the culture was grown to an $O.D._{600}$ of 0.4 and then placed on ice. 0.1 ml of the culture and 0.9 ml of Z buffer (0.06 M Na_2HPO_4, 0.04 M NaH_2PO_4, 0.01 M KCl, 0.001 M $MgSO_4$, 0.05 M β-mercaptoethanol) were mixed together with two drops of chloroform and 1 drop of 0.1% SDS. The solution was vortexed for 10 seconds and then equilibrated to 28°C. 0.2 ml of *o*-nitrophenyl-β-D-galactopyranoside (4 mg/ml in A medium) was added to the culture and incubation continued until a yellow color developed. The reaction was stopped by the addition of 0.5 ml of 1 M Na_2CO_3.

The reaction samples were read in a spectrophotometer at 420 nm and at 550 nm. The β-galactosidase activity was assessed in Miller units, according to the following formula:

$$1000 \times \frac{A_{420} - (1.75 \times A_{550})}{t \times 0.1 \times A_{600}}$$

Where:

t = time to color change

23. Incompatibility testing

Incompatibility was tested by introducing one plasmid (by transformation or conjugation) into a strain harboring the second plasmid. Transformants or transconjugants were selected for the presence of the incoming plasmid only or for both plasmids simultaneously, transferred to LB and incubated without selection for 18-24 hours at 37°C. After incubation, the culture was diluted in phosphate buffer (pH 7.2) and various dilutions were plated on selective media. The percentage of cells harboring each plasmid separately and both together was determined by comparing colony counts on selective media.

24. Copy number tests

Plasmid copy number was estimated by isolating plasmid DNA from strains harboring the plasmid of interest. Cells were grown in LB at 37°C with antibiotic selection for 16 - 18 hours and diluted 1:50 in fresh media. Following an additional 2 hour growth period, total DNA was isolated from 1 ml of the culture by the following method. Cells were subjected to centrifugation in an Eppendorf microfuge at maximum speed for 5 minutes. The cell pellet was resuspended in 150 µl of buffer (50 mM Tris, pH 8.0, 10 mM EDTA, 25% sucrose, 5 mg/ml lysozyme), and incubated at 37°C for 30 minutes. The mixture was extracted with phenol-chloroform, and nucleic

acids were precipitated from the aqueous phase with ethanol. Live colony counts were evaluated by plating culture dilutions on L agar. Quantities of DNA were spotted onto nitrocellulose filters using a dot blot apparatus. DNA isolated from a strain carrying R27 was used as a standard for comparison. The blots were denatured and neutralized as described, and a radioactively-labelled DNA fragment encompassing a portion of the minimal replicon was hybridized with the blot. Dot intensities of the test plasmids were compared with the R27 standard in order to estimate copy number.

25. Stability tests

Plasmid stability was determined in the absence of antibiotic selection. Plasmid-bearing strains were initially grown in broth overnight at 37°C in the presence of an antibiotic required for plasmid selection. A 1:1000 dilution of the culture was made with fresh media, and growth allowed to continue without selection at 37°C for an additional 7 -14 hours. This step was repeated several times. At each dilution step, the culture was plated onto selective and non-selective agar, and viable counts were determined. Stability was defined as the percentage of cells which retained the resistance determinant at each dilution step.

26. Sequenase sequencing

Double-strand plasmid DNA template was used for nucleotide sequence determination. Sequencing of plasmid DNA was accomplished by

the dideoxynucleotide termination method as described by Sanger *et al.*(122) using [^{35}S]dATP and a Sequenase (141) kit (United States Biochemical), and carried out according to the manufacturer's instructions. Plasmid DNA was either chemically denatured (163) or thermally denatured (by heating to 97°C) prior to annealing with an appropriate primer. Synthetic oligonucleotides were used as primers. Sequencing products were separated in 6-7% polyacrylamide gels.

27. Taq sequencing

Cycle sequencing employing Taq polymerase was carried out as follows. Synthetic oligonucleotides used as primers were end-labelled with [γ-^{33}P]ATP using T4 polynucleotide kinase. For each sequencing reaction, 1 pmol of primer was mixed with 2 pmol radiolabelled ATP in a total volume of 5 µl of kinase buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 5 mM DTT, 0.1 mM spermidine, pH 8.2). The mixture was incubated at 37°C for 10 min., then at 55°C for an additional 5 min to inactivate the kinase. The labelled primer was placed on ice for use in the sequencing reaction.

15 - 50 fmol of each template plasmid was added to 5µl of labelled primer, along with 4.5 µl 10X Taq sequencing buffer (300 mM Tris-HCl, pH 9.0, 50 mM $MgCl_2$, 300 mM KCl, 0.5% W-1) and 2.5 U of Taq DNA polymerase, and the total volume adjusted to 36 µl with distilled water. For each termination reaction, 8 µl of this mixture was added to 2 µl of

termination mix (see Table II-4 for composition of termination mixes), and overlaid with one drop of mineral oil. All reaction tubes were transferred to a Perkin-Elmer 960 thermocycler for incubation. Reactions were incubated for 40 cycles with the following parameters: 94°C, 30 sec.; 50°C, 40 sec.; 72°C, 45 sec.

Table II-4 Composition of cycle sequence termination mixtures

Termination Mixture	Composition
A	2 mM ddATP, 100 μM each of dATP, dCTP, d GTP, dTTP
C	1 mM ddCTP, 100 μM each of dATP, dCTP, d GTP, dTTP
G	0.2 mM ddGTP, 100 μM each of dATP, dCTP, d GTP, dTTP
T	2 mM ddTTP, 100 μM each of dATP, dCTP, d GTP, dTTP

28. Mini-Tn10 transposon insertion mutagenesis

The mini-Tn10 derivative plasmid pNK2884 was introduced by transformation into a strain carrying the transfer-derepressed R27 derivative pDT1942. Transformants were transferred to L-broth and incubated at 28°C for several hours with agitation, until an optical density at 600 nm of 0.15 - 0.30 was reached. IPTG was then added to a final concentration of 1 mM, and incubation continued for an additional 2 hours. A portion of this culture

was then used as a donor in a 1 hour conjugation with an appropriate recipient. Transconjugants carrying antibiotic resistance markers of pDT1942 (Tc, Km) and of the mini-Tn10 (Cm) were selected for. Each transconjugant carrying the appropriate antibiotic resistance pattern was tested for conjugational proficiency in order to select Tra$^-$ derivatives for further study.

29. Pili purification

Purified conjugative pili were prepared using a modification of the method described by Armstrong *et al.* (11). A single isolated colony of the desired strain was transferred to a 5-ml BHI culture and incubated overnight at 28°C, without agitation. The following day, 1 ml of this culture was transferred to 150 ml of fresh BHI broth, and incubated at 28°C for an additional 3 - 5 hours. This culture was then used to inoculate aluminum trays containing DST agar (with appropriate antibiotics). The trays were incubated overnight at 28°C. The next day, cells were harvested by adding 20 mls of 1X SSC to each tray and scraping growth from the agar.

Harvested cells were gently agitated at 4°C overnight to remove the pili from the cells and the cells and debris were separated from the free pili by two separate rounds of centrifugation at 10,000x*g* for 20 min. A concentrated solution of ammonium sulfate was then added drop-wise to the supernatant to a final concentration of 15% (w/v). After resting overnight at

4°C, the precipitated pili were collected by centrifugation at 10,000xg for 20 min. The precipitate was resuspended in 5 - 10 ml of Milli-Q water, and insoluble material was removed by centrifugation at 10,000xg for 10 min. To purify the pili further, additional rounds of ammonium sulfate precipitation were carried out. Finally, the purified pili were dialyzed extensively against milli-Q water to remove traces of ammonium sulfate.

In some cases an additional step was added as follows. Crude pili preparations were dialyzed against milli-Q water to remove traces of ammonium sulfate, and cesium chloride added to adjust the density of the solution 1.3 g/ml. The solution was then subjected to centrifugation in a Beckman SW-55 rotor at 38,000 RPM for 20 - 24 hours at 5°C. After centrifugation, bands of protein were carefully removed from the centrifuge tube, transferred to dialysis tubing, and dialyzed against milli-Q water to remove cesium chloride from the purified protein.

30. Electron microscopy

Samples were prepared for electron microscopy as follows. Cell suspensions (approx. 10^8 CFU/ml) were prepared in LB or in phosphate buffer (pH 7.2). Cells or protein preparations were spotted onto formvar coated copper grids (200 mesh), allowed to settle for 1 - 2 min. and blotted. Samples were stained with 1% sodium phosphotungstate, and examined using a Phillips 300 transmission electron microscope.

31. Amino acid analysis and N-terminal sequencing

Amino acid analysis of purified H pili was derived from pili hydrolyzed in 6M HCl under vacuum at 110°C for 24 hours (107). Cysteine and methionine residues were identified after performic acid oxidation of pilin protein. Hydrolyzed protein was analyzed using a Beckman 6300 analyzer (Beckman Instruments, Inc.). Amino-terminal sequencing was carried out by automated Edman degradation in the presence of polybrene. N-terminal sequencing was attempted with purified protein and from pilin protein blotted to PVDF membrane.

32. Proteolytic digestion of pilin protein

Purified pilin protein was subjected to enzymatic digestion using a number of different proteases. The protein was dissolved in denaturant (6M guanidine-HCl or 8M urea, 50 mM Tris, pH 8.0, 5 mM DTT) and heated to 95°C for 15-20 min., or to 60°C for 45-60 min. After allowing the mixture to cool to room temperature, a reaction buffer (see Table II-5) was added to bring the guanidine-HCl or urea concentration to less than 1 M.

An appropriate quantity of protease was added to the denatured pilin solution (to a final molar ratio of 1:200 to 1:10, protease:pilin). The digestion was allowed to proceed for up to 24 hours at 37°C. After digestion, the sample was precipitated by the addition of TCA to a 10% final concentration,

and precipitated peptides resuspended in PBS. Samples were analyzed by SDS-PAGE and reverse phase HPLC.

Table II-5 Buffer components for protease digestion

Protease	Buffer Components[a]
Endoproteinase Glu-C	50 mM ammonium acetate, pH 4.0 **OR**
	50 mM ammonium bicarbonate, pH 7.8
Modified Trypsin	50 mM NH_4HCO_3, pH 7.8 **OR**
	50 mM Tris-HCl, 1 mM $CaCl_2$, pH 7.6

[a]Both buffers listed for each enzyme were tested separately

33. Antibody preparation

Antisera to purified pili were raised in specific-pathogen-free New Zealand White rabbits. Samples of purified pili (5 - 10 μg) were prepared and mixed with 50 μg of Quil-A Saponin (Superfos Biosector a/s) prior to injection. Subcutaneous injections at multiple sites were performed several times at two to three week intervals. At the second and subsequent injections, 3 - 5 ml of blood were collected for analysis by ELISA to determine antibody titre. After adequate antibody titre was obtained, blood was collected by cardiac puncture. After clotting, sera were separated from cellular components by centrifugation at 2,000x*g* for 15 min. The sera were aliquotted in small quantities and stored at -80°C for further use.

34. Absorption of antisera with acetone powders

Acetone powders of *E. coli* strains were prepared as follows. An overnight culture of the selected bacterial strain was diluted 1:100 in fresh media (BHI or LB) and incubated with aeration at 28°C or 37°C until the culture reached an $O.D._{600}$ of 0.6 - 0.7. Cells were recovered by centrifugation (Beckman JA-20 rotor, 5000 RPM, 15 min.) and resuspended in 0.9% NaCl (1 ml per gram of wet cell weight). After resting on ice for 5 min., 8 ml of acetone (-20°C) per 2 ml of cell suspension was added and the solution mixed vigorously. The mixture was then incubated on ice for 30 min. with occasional mixing. After subjecting the mixture to centrifugation (10,000x*g*, 10 min.), the cell pellet was transferred to a clean piece of filter paper and allowed to dry. The dried pellet was dispersed to a fine powder and transferred to an airtight container for long term storage.

Acetone powders were used in antisera absorption by adding the powder to a final concentration of 1% (w/v) directly to the unabsorbed antisera. The mixture was incubated on ice for 30 min., subjected to centrifugation (10,000x*g*, 10 min.), and the supernatant carefully removed for further use.

35. Western blot

SDS-PAGE gels were blotted to nitrocellulose filters with a tank electroblotting device (BioRad, Inc.) using a 25 mM phosphate buffer (pH

7.4). After electrophoretic transfer (27V, 4 - 12 hours), the nitrocellulose membrane was transferred to a container and blocking buffer added (TBS, 3% BSA, 5% skim milk powder, 0.05% Tween-20, pH 7.2) to cover the blot. The membrane was incubated at room temperature for 1 - 2 hours, or overnight at 4°C. After blocking, the buffer was removed and the membrane washed three times (5 min. each) in washing solution (TBS, pH 7.2). The primary antibody was diluted in blocking buffer, added to the blot, and incubated at room temperature for 2 hours. After draining the primary antibody solution, the blot was washed six times (5 min. each) in washing solution. The secondary antibody (horseradish peroxidase- or alkaline phosphatase-conjugated goat anti-rabbit IgG, Boehringer Mannheim Chemicals, Ltd.) was diluted in blocking buffer and added to the blot. After incubating at room temperature for 2 hours, the blot was again washed six times in washing solution.

Conjugated antibodies were detected either by color development or by enhanced chemiluminescence. For colorimetric detection (alkaline-phosphatase conjugate), 1 ml of NBT stock solution (*p*-nitro blue tetrazolium chloride, 50 mg/ml in 70% N,N,dimethylformamide) and 1 ml of BCIP stock solution (5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt, 25 mg/ml in N,N,dimethylformamide) were mixed together in 98 ml of carbonate buffer (0.1 M $NaHCO_3$, 1 mM $MgCl_2$, pH 9.8). This developer solution was added to

the blot, and color development allowed to proceed for several minutes. The reaction was stopped by rinsing the blot in distilled water.

For chemiluminescent detection, an enhanced chemiluminescence system (Amersham, Buckinghamshire, England) was used in accordance with the manufacturers directions. Following treatment with the chemiluminescence reagents, blots were exposed to X-Ray film (Kodak X-Ar) for various time periods (15 sec. - 20 min.).

36. ELISA

Pili were diluted to approximately 2.5 μg/ml in 0.5M Na_2CO_3 and 150 μl was dispensed into wells of a microtitre plate (PVC high activity). The plate was stored at 4°C overnight to allow coating of antigen to the walls of the tray. The fluid was aspirated from the wells of the tray, and washed three times with washing solution (PBS, 0.1% Tween-20, 0.004% Thimersol). 100 μl of blocking buffer (PBS, 1% BSA, 0.1% Tween-20, 0.004% Thimersol) was added to each well and incubated at room temperature for 2 hours. After removing the blocking buffer, wells were washed three times with washing solution. Test sera was diluted 1:100 in serum diluent (PBS, 0.5% BSA, 0.1% Tween-20, 0.004% Thimersol) and 100 μl of control and test sera were added to wells in a two-fold dilution series. A row of control blanks was also included (dilution buffer only). The tray was then incubated at room temperature for 2 hours.

After removing the primary antisera solutions (+ controls) and washing three times with wash solution, 100 µl of a 1:2500 dilution of secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG, Boehringer Mannheim Chemicals Ltd.) was added to each well. The tray was then incubated at room temperature for 2 hours. After aspirating the secondary antibody, the tray was washed three times with washing buffer, and three additional times with a solution of PBS and 0.004% thimersol. 100 µl of horseradish peroxidase substrate (1 mM 2,2'-azino-di-[3-ethyl-benzthlazoline sulfonate], 0.03% H_2O_2, 25 mM citrate buffer, pH 4.2) was added to each well and incubated at room temperature for 15 min. After color development, the reaction was stopped with the addition of 50 µl of stop solution (0.001% sodium azide, 0.1 M citric acid). The plates were scanned at 405 nm with a Titertek Multiscan (Flow Laboratories, Mclean, Virginia) and results expressed as an average of two readings.

37. Pulse-chase labelling of pilin protein

Overnight cultures of the appropriate *E. coli* strain (grown in LB) were diluted to an $O.D._{600}$ of 0.1 - 0.2 in M9 medium, supplemented with the necessary amino acids and vitamins. Incubation of the culture was continued with aeration until the cell density reached an $O.D._{600}$ of 0.4. The radiolabelled amino acid was added (10 µCi/ml) and incubation continued for one min. (or longer in some cases). A sample of the culture was collected at

the end of the pulse phase, and the culture diluted with two volumes of fresh M9 media containing unlabelled amino acid (2 mM). In some experiments, chloramphenicol was added at this point to a final concentration of 26 μg/ml. Incubation was continued and samples were collected at various time points after the cold chase was added. Cells in each sample were collected by centrifugation (14,000x*g*, 30 sec.), resuspended in SDS-PAGE cracking buffer (62.5 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.00625% BPB), and heated to 97°C for 3-5 min. Samples were subjected to high-resolution electrophoresis in SDS polyacrylamide gels. After electrophoresis, gels were soaked in 1M sodium salicylate for 30 min., dried, and exposed to X-ray film (Kodak X-Ar) for various time periods.

38. Phage sensitivity testing

The H pilus-specific bacteriophage pilHα was used in plaquing experiments for the detection of pilus production as follows. Appropriate *E. coli* strains, harboring R27 or a derivative thereof, were grown overnight in BHI broth at 28°C. The culture was diluted 1:100 with fresh media and incubation continued for an additional 4 hours. Following this, 100 μl of a 1:5 dilution (diluted in fresh BHI, prewarmed to 28°C) of the culture was mixed with 1.5 ml of BHI + 0.6% agar (equilibrated at 40°C). The suspension was quickly spread onto a BHI agar plate (containing appropriate selective antibiotics) and allowed to solidify. One drop of a high titre phage

suspension (approx. 2.0 x 10^{10} PFU/ml) was spotted onto the agar, and the plate incubated for 16 - 20 hours at 28°C. Plates were examined for zones of lysis.

Chapter III RepHI1A - a minimal replicon of R27[1]

A. Subcloning of an autoreplicative fragment from pDT1233

Plasmid pDT1233 consists of a 32-kb fragment of R27 cloned into the *Sal*I site of the cosmid vector pHC79 (see Figure I-2). This plasmid was capable of transforming *E. coli* strain SF800 (*polA*) with high efficiency, whereas the cosmid vector itself (pHC79) could not transform this host. This implied the presence of a PolI-independent replicon within the cloned fragment. pDT1233 was subcloned as smaller fragments in order to identify the location of the PolI-independent replicon within the 32-kb region. Several subclones were able to replicate in the *polA* host SF800. The smallest of these contained a 2.4-kb *Sma*I/*Xba*I fragment. The results of this subcloning analysis are presented in Figure III-1.

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[1] A version of this chapter has been published. **Newnham, P. J., and Taylor, D. E.** 1994. Molec. Microbiol. 11:757-768

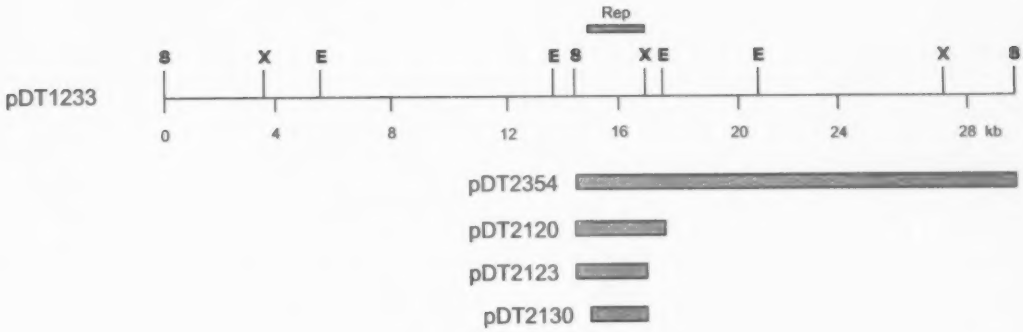


Figure III-1 Subcloning analysis of pDT1233.

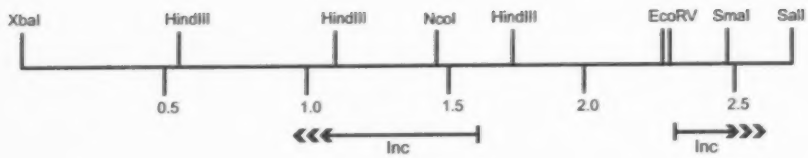
pDT1233 is a cosmid subclone of R27 encompassing co-ordinates 22.2 - 54.2 on the R27 map (total size of R27 = 182 kb). Restriction sites indicated are as follows: E, E*co*RI; X, X*ba*I; S, S*al*I. Subclones of pDT1233 are indicated below the map. Only those subclones capable of transforming the *E. coli polA* host SF800 are shown. The smallest region common to all subclones is indicated above the map.

B. Deletion analysis of replicon.

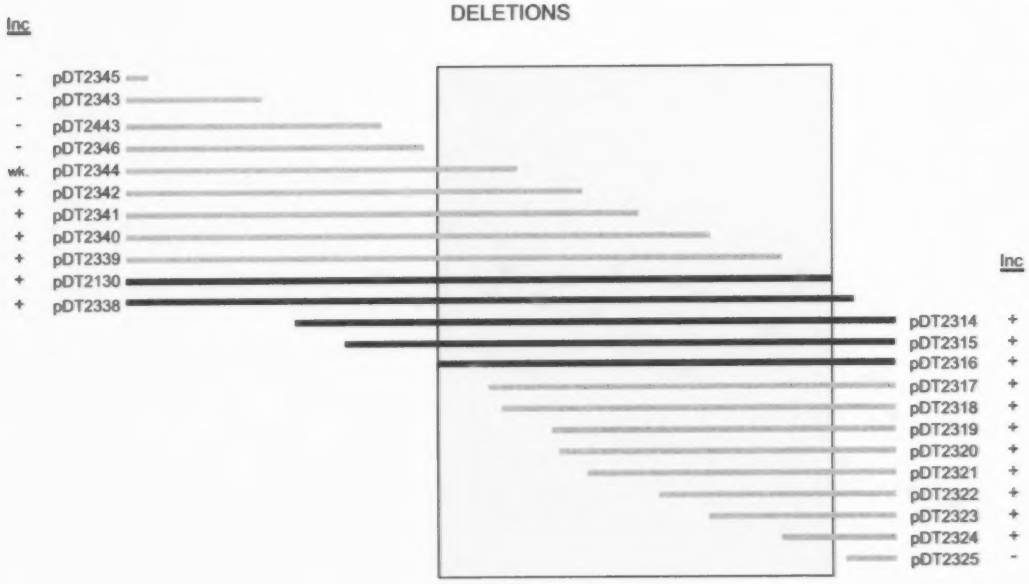
Plasmid pDT2123 consists of a 2.7-kb S*al*I/X*ba*I fragment of pDT1233 cloned into the multicloning site of pUC13, and harbours a functional PolI-independent replicon. As a means of determining the sequence of the minimal replicon encoded within pDT2123 and to aid in delineating the physical limits of the replicon, unidirectional deletions were constructed from both ends of the fragment (see Figure III-2). In order to generate Exonuclease III deletions from the X*ba*I site directed into the cloned fragment, the plasmid was first digested with E*co*RI and S*st*I prior to ExoIII digestion. For deletions from the S*al*I site, pDT2123 was digested with P*st*I and S*al*I. Each deletion mutant constructed was tested for its ability to replicate in the *E. coli polA* host SF800 by transformation. In all cases where SF800 was used to determine replication proficiency, pUC13 was used as a control in the transformation experiment. Ampicillin-resistant transformants were never observed with the vector alone. Deletion analysis defined a minimal replicon of approximately 1.4-kb in size.

Figure III-2 Mapping and deletion analysis of the minimal replicon.

The restriction map of the 2.7-kb S*al*I/X*ba*I fragment harbouring the minimal replicon of R27 (pDT2123) is shown. A series of deletion mutants, constructed as described, are depicted below the map as bars which represent material remaining after digestion with Exonuclease III. Black bars represent deletions capable of replicating in the *polA* host SF800; shaded bars are shown for those that failed to transform this host. Incompatibility phenotypes for each deletion are indicated on the left and right sides as follows: +, strong incompatibility; -, no incompatibility; wk., weak incompatibility. The incompatibility patterns exhibited by the various deletions define the endpoints of at least two distinct regions that contribute to incompatibility, as shown on the map. The minimal replicon defined by deletion analysis is boxed and shaded.


XbaI
HindIII
HindIII
NcoI
HindIII
EcoRV
SmaI
SalI
0.5
1.0
1.5
2.0
2.5
Inc
Inc

DELETIONS


Inc
- pDT2345
- pDT2343
- pDT2443
- pDT2346
wk. pDT2344
+ pDT2342
+ pDT2341
+ pDT2340
+ pDT2339
+ pDT2130
+ pDT2338
Inc
pDT2314 +
pDT2315 +
pDT2316 +
pDT2317 +
pDT2318 +
pDT2319 +
pDT2320 +
pDT2321 +
pDT2322 +
pDT2323 +
pDT2324 +
pDT2325 -

C. Incompatibility phenotypes of the minimal replicon

Incompatibility could not be observed between pDT2123 and R27 using DH5α as the host strain. Both plasmids were co-maintained without selection for greater than 100 generations, without appreciable loss. In order to investigate this unusual phenomenon further, a plasmid was constructed which consisted of the same 2.7-kb S*al*I/X*ba*I fragment present in pDT2123 ligated to a kanamycin resistance cassette (pKIXX, Pharmacia). This plasmid (pDT2376) carried no vector origin of replication and thus could only replicate from the cloned R27 origin. An extremely potent incompatibility reaction was apparent between pDT2123 and pDT2376, such that the two plasmids could never be simultaneously selected for in the same host. In addition, each of the deletion mutants (see Figure III-2), and several PCR-amplified fragments (see below) derived from pDT2123 were tested for incompatibility with pDT2376. Since incompatibility could not be tested directly due to the inability of both pDT2376 and pDT2123 to be simultaneously selected for, an altered incompatibility test was devised. Each combination (deletion construct + pDT2376) was selected for the incoming plasmid alone (Ap resistance) and for both plasmids together (Ap + Km resistance). Incompatibility was scored positive if transformants appeared under Ap selection but not under Ap + Km selection. Incompatibility was scored as negative if transformants appeared under both single and double selection. As shown in Figure III-2, the deletions define one border of at least

two regions of pDT2123 that are responsible for the observed incompatibility reactions.

D. Nucleotide sequence of R27 minimal replicon.

The complete DNA sequence of the replicon contained in pDT1233 was determined by double stranded sequencing. Both DNA strands were sequenced to eliminate potential errors. The 1747-bp sequence is shown in Figure III-3.

A single large open reading frame (ORF) is present within the sequence (nucleotides 497 - 1372), encoding a 33-kDa protein. Two additional potential start codons lie further downstream within the same ORF (GTG - position 533; GTG - position 632) and would encode 32-kDa and 28-kDa proteins, respectively. None of these potential start codons is preceded by a consensus Shine-Dalgarno ribosome binding site (128).

Several structures characteristic of *E. coli* origins of replication are located upstream of the large ORF. Two 9-bp sequences (nt 227-235, 236-244) located next to one another conform to the consensus sequence for DnaA binding (44). Each has a single mismatch from the consensus. In addition, five GATC sequences, the recognition sequence of DNA adenine methylase (47), are located between positions 250 and 341. Four of these are embedded in a 7-bp repeating motif (AGATCAA).

A total of seven repeated oligonucleotides with the consensus sequence AAAAGCATTTGnATGAATG are present in the minimal replicon (Table III-1). Four of these are found in tandem, upstream of the large ORF (Figure III-3). Three additional repeats of this class are located downstream of the ORF and are oriented in the opposite direction (Figure III-3). All seven repeats have no more than four mismatches from the consensus sequence. In addition, a number of sequences near or within both the upstream and downstream repeat structures appear to be related to the core repeat consensus, but are considerably more degenerate (not shown).

The sequence reported here has been submitted to the Genbank Nucleotide Sequence Data Library, and appears under accession number M95772.

Figure III-3 DNA sequence of the minimal replicon.

The DNA sequence of 1747 bases encompassing the minimal replicon is shown. The putative RepA orf is translated below the sequence. Unshaded, boxed sequences represent the two DnaA binding sites (1 mismatch each from the consensus sequence). Shaded, boxed sequences indicate Dam methylase recognition sequences upstream of the orf. The large arrows beneath the sequence are 19-bp iterons; the direction of the arrowhead indicates the orientation of the iteron. Two overlined regions (nt 394-399, 415-420) indicate probable -35 and -10 *E. coli* promoter elements, as defined by primer extension. The 5' ends of mRNA encoding the RepA protein are indicated by the rightward pointing arrows (defined by primer extension). The large arrow represents the main extension product, while the smaller arrow shows the minor extension product.

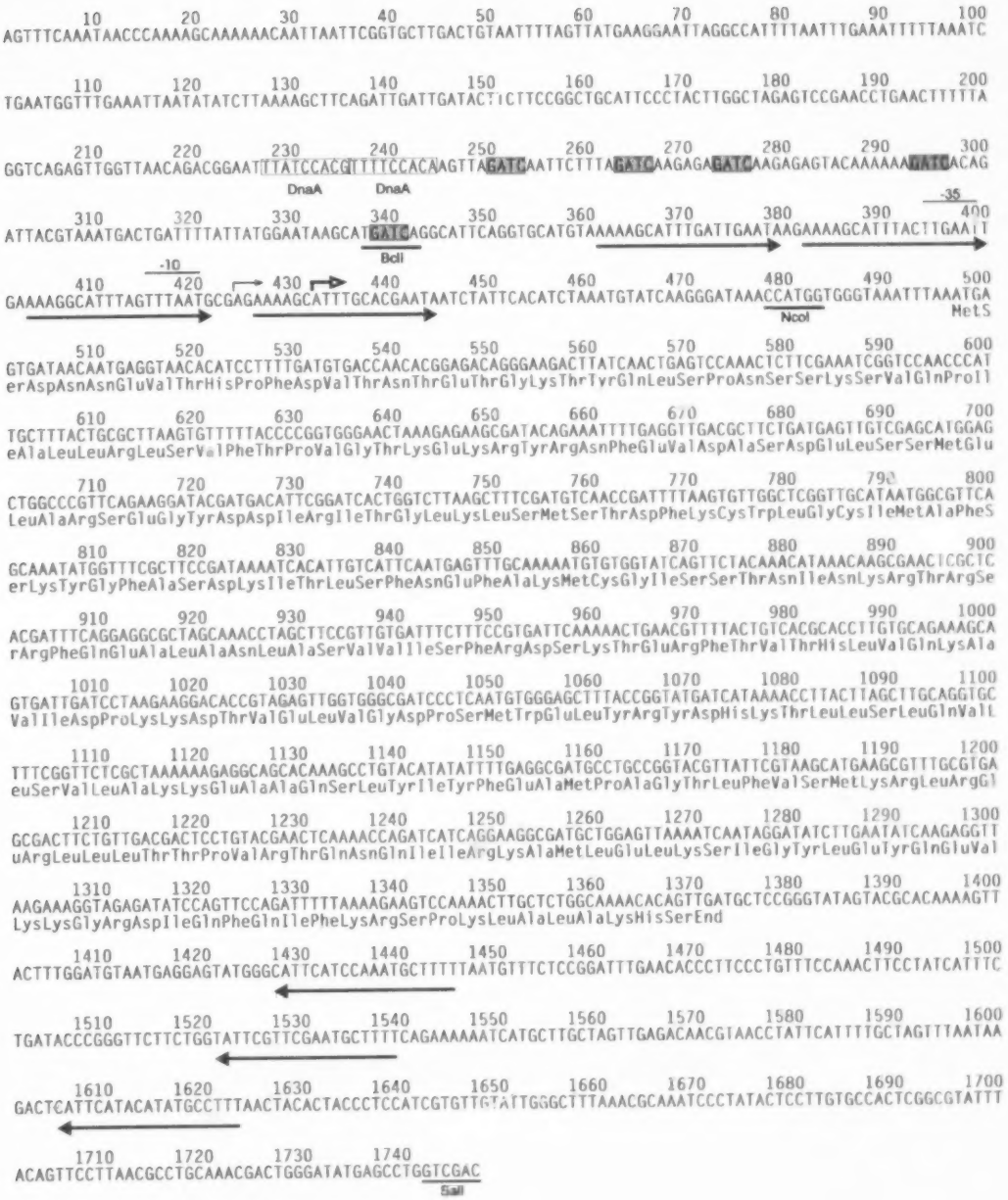

DnaA
DnaA
BclI
-35
-10
NcoI
MetS
SalI

Table III-1 Iterons in the minimal replicon of R27

Iteron[a]	Orientation[b]	Sequence[c]
1	→	AAAAGCATTTGATTGAATA
2	→	AAAAGCATTTACTTGAATT
3	→	AAAGGCATTTAGTTTAATG
4	→	AAAAGCATTTGCACGAATA
5	←	AAAAGCATTTGGATGAATG
6	←	AAAAGCATTCGAACGAATA
7	←	AAAGGCATATGTATGAATG
Consensus		AAAAGCATTTGnATGAATG

[a]Iterons are indicated in Figure III-3. They are numbered in order with 1 representing the furthest upstream iteron, 7 indicating the furthest downstream iteron (with respect to the ORF).

[b]Orientation is either the same as the ORF (→) or opposite to that of the ORF (←).

[c]Sequences are shown 5'→ 3'.

E. Polypeptides encoded in the minimal replicon

Several replication-proficient constructs were used in an *in vitro* transcription-translation experiment to examine protein production. Figure III-4 depicts the results of this experiment. A faint band with an apparent molecular weight of 34-kDa is present in both of the constructs examined (pDT2316 and pDT2315). This band is completely absent from the control lane (pUC13). This protein has been designated RepA, since deletion analysis has shown it to be required for replicative proficiency of the replicon. The apparent molecular weight of this protein is in good agreement with the deduced molecular weight of the Rep protein (33-kDa), as determined from the DNA sequence. Synthesis of the protein is limited to small quantities, when compared with vector encoded polypeptides. No other polypeptide products could be detected that were present in replicon subclones and absent in vector controls, suggesting that the RepA protein is the only polypeptide produced by the minimal replicon.

In addition to the *in vitro* analysis, the putative coding sequence of the RepA protein was ligated into the multicloning site of the expression vector pMS119. This vector carries the strong *tac* promoter upstream of the multicloning site, providing efficient transcription of DNA cloned into the vector, which is inducible by the addition of IPTG. The presence of a 34-kDa protein could easily be detected in cells harboring this construct (pDT2536), after induction with 1 mM IPTG, as shown in Figure III-5.

Figure III-4 *In vitro* transcription-translation of minimal replicon-encoded proteins.

Proteins were synthesized and labelled with [^{35}S]-methionine as described. Labelled proteins were subjected to electrophoresis on a sequencing gel apparatus using a 12% SDS-polyacrylamide gel, in order to optimize separation of vector-encoded and replicon-encoded proteins. The position of the 30-kDa molecular weight marker is indicated (none of the remaining markers are visible in this photograph). The small arrowhead to the right indicates the location of the RepA protein. Lane 1 - no template DNA; lane 2 - pUC13; lane 3 - pDT2316; lane 4 - pDT2315.

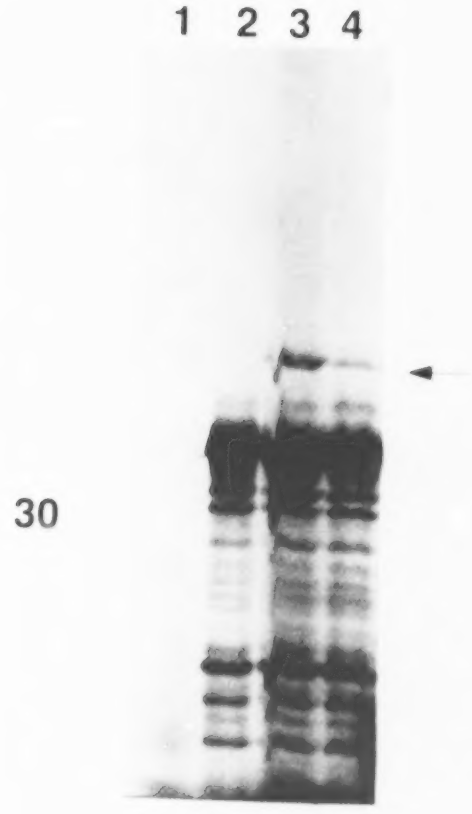
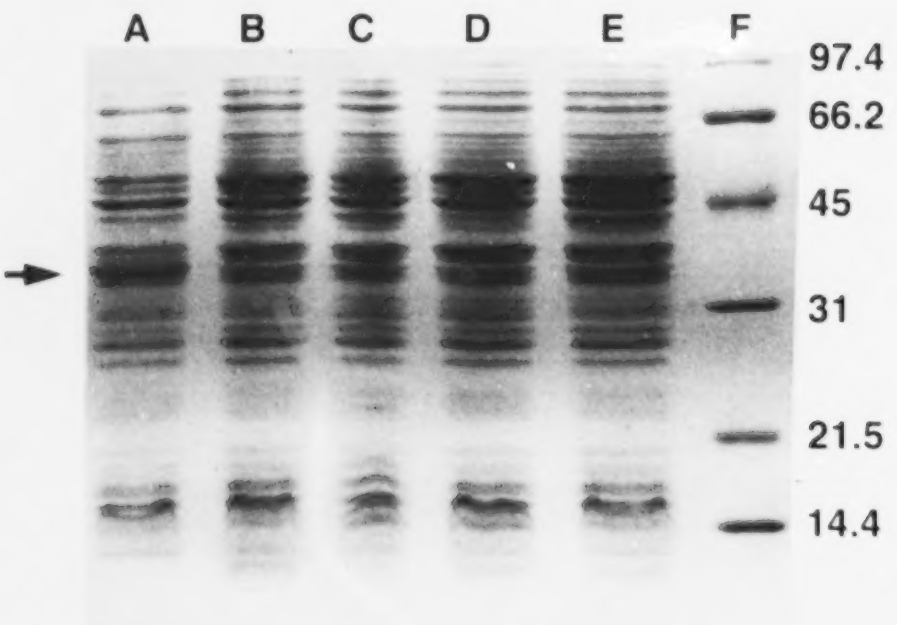

1 2 3 4
30

Figure III-5 Expression of RepA from the *tac* promoter.

Protein samples were prepared from whole cells, as described, subjected to electrophoresis in 12% SDS polyacrylamide gels, and stained with Coomassie Blue. Lane A - pDT2536 (pMS119 + *repA* coding sequence), induced with 1 mM IPTG; lane B - pDT2536, no induction; lane C - pMS119, induced with 1 mM IPTG; lanes D and E - pMS119, no induction; lane F - molecular weight standards. The sizes of the molecular weight standards are indicated in kDa to the right of the photograph. The over-expressed RepA protein is indicated by an arrow.

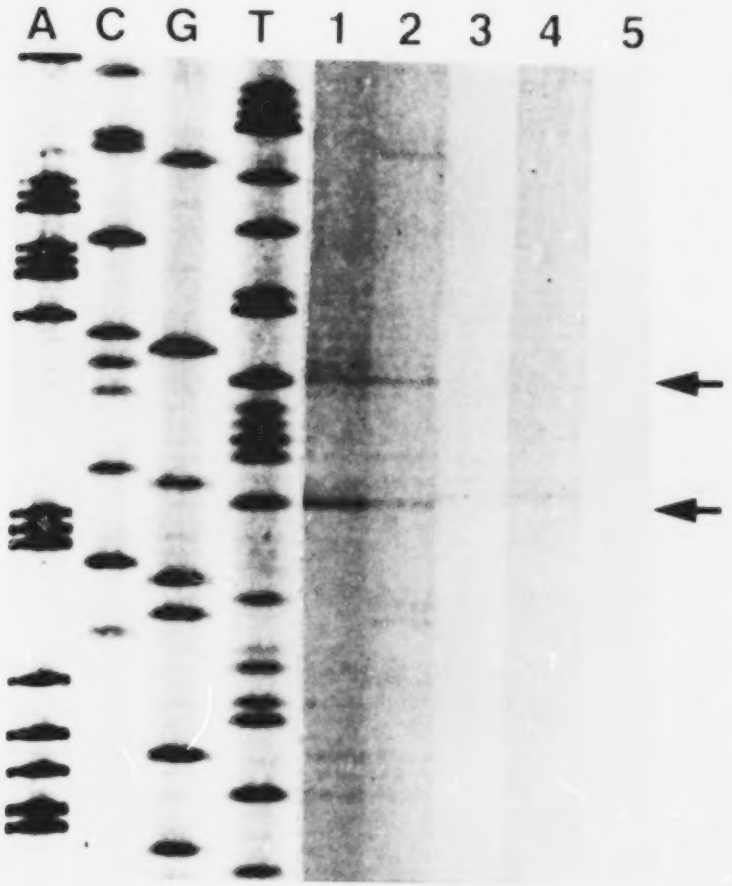

A
B
C
D
E
F
97.4
66.2
45
31
21.5
14.4

F. Promoter mapping

Primer extension analysis was used to map the 5' end of the transcript encoding the putative replication initiator. The primer used (5'-CTTAAGCGCACTAAAGC-3') was homologous to nt 601 - 618, 105 nt downstream of the putative start codon of the Rep ORF. A sequence ladder generated with the same primer was used for comparison. Unique extension products could not be observed when RNA produced from Rep^+ subclones of R27 was used as the template for the reaction. However, RNA isolated from two deletion derivatives of pDT2123 (pDT2339 and pDT2341) generated two faint extension products, as shown in Figure III-6. These bands correspond with nt 425 and 432 respectively. The band at position 432 is more prominent. Sequences bearing similarities to *E. coli* promoters are present upstream of these positions. These include nt 394 - 399, which bear a likeness to the -35 promoter consensus, and nt 415 - 420, similar to the -10 promoter consensus (52). These two regions are separated by 15 nucleotides. This promoter structure is 5 and 12 nt upstream of the 5' ends of the Rep transcript identified by primer extension.

Figure III-6 Primer extension analysis.

RNA was isolated and primer extension was carried out as described. The primer used (5'-CTTAAGCGCACTAAAGC-3') is homologous to nt 601 - 618, 105 bp downstream of the putative start codon of the RepA protein. Lanes A, C, G, T - dideoxy-terminated sequence using the same primer and pDT2123 as template. Lane 1 - pDT2339 (Rep^-); lane 2 - pDT2341 (Rep^-); lane 3 - pDT2315 (Rep^+); lane 4 - pDT2339 (no primer); lane 5 - DH5α (no plasmid). Major and minor extension products are indicated with arrows. Lanes showing extension products were exposed to X-ray film for 7 days to enhance their visibility, while the sequence ladder was exposed for 1 day.


A
C
G
T
1
2
3
4
5

G. Assay of promoter activity

A promoter probe vector, pJL207 (7, 25), was used to analyze the activity and regulation of the promoter responsible for the synthesis of the RepA protein. This vector contains an open reading frame which encodes β-galactosidase as well as appropriate translation initiation signals. A multi-cloning site is present upstream of the *lacZ* gene which allows DNA fragments to be easily inserted into the vector. When inserted in the proper orientation, a DNA fragment carrying an active promoter results in the expression of β-galactosidase, which can be quantitatively assayed. For our purposes, a 146-bp fragment from the minimal replicon (from the unique B*cl*I site to the N*co*I site - see Figure III-3) was ligated into the multi-cloning region of pJL207. The resulting construct, pDT2531, produced red colonies when transformed into *E. coli* strain DH5α and plated on MacConkey agar. Constructs in which the insert was cloned in the opposite orientation resulted in white colonies on MacConkey agar.

β-galactosidase activity was measured by the method of Miller (91). Cells were grown in L broth and β-galactosidase activity assessed as described. All plasmids tested were assayed using DH5α as the host strain. As well as evaluating the baseline activity of the *repA* promoter, the effect of RepA protein on the transcriptional activity of the promoter was examined by introducing a second plasmid which encoded RepA. The results of this analysis are shown in Table III-2. Plasmid pDT2536 consists of the coding

sequence of the *repA* gene ligated to the expression vector pMS119 in such a manner as to allow high-level synthesis of the RepA protein by induction with IPTG. This plasmid and pDT2534 were used as sources of RepA in the experiment. As can be seen from the data, the presence of plasmid pDT2534 or pDT2536 results in a 33- to 40-fold reduction in β-galactosidase activity, when compared to control plasmids (pUCBM20 and pMS119).

Table III-2. Promoter activity of *repA* promoter

Plasmid(s)[a]	RepA *in trans*[b]	β-galactosidase Activity[c] (Miller Units)
pJL207	N.A.	7.83 ± 0.11
pDT2531	N.A.	185 ± 7
pDT2531 + pDT2534	+	4.93 ± 0.20
pDT2531 + pUCBM20	-	200 ± 7
pDT2531 + pDT2536	+	5.57 ± 0.26
pDT2531 + pMS119	-	185 ± 12
pDT2534	N.A.	< 0.01
pUCBM20	N.A.	0.14 ± 0.04
pDT2536	N.A.	< 0.01
pMS119	N.A.	0.08 ± 0.07

[a]In each case, the *E. coli* host DH5α was used for the assay.

[b]Signifies whether the co-resident plasmid carries an intact *repA* gene: +, *repA* present on co-resident plasmid; -, *repA* not present on co-resident plasmid; NA, no co-resident plasmid.

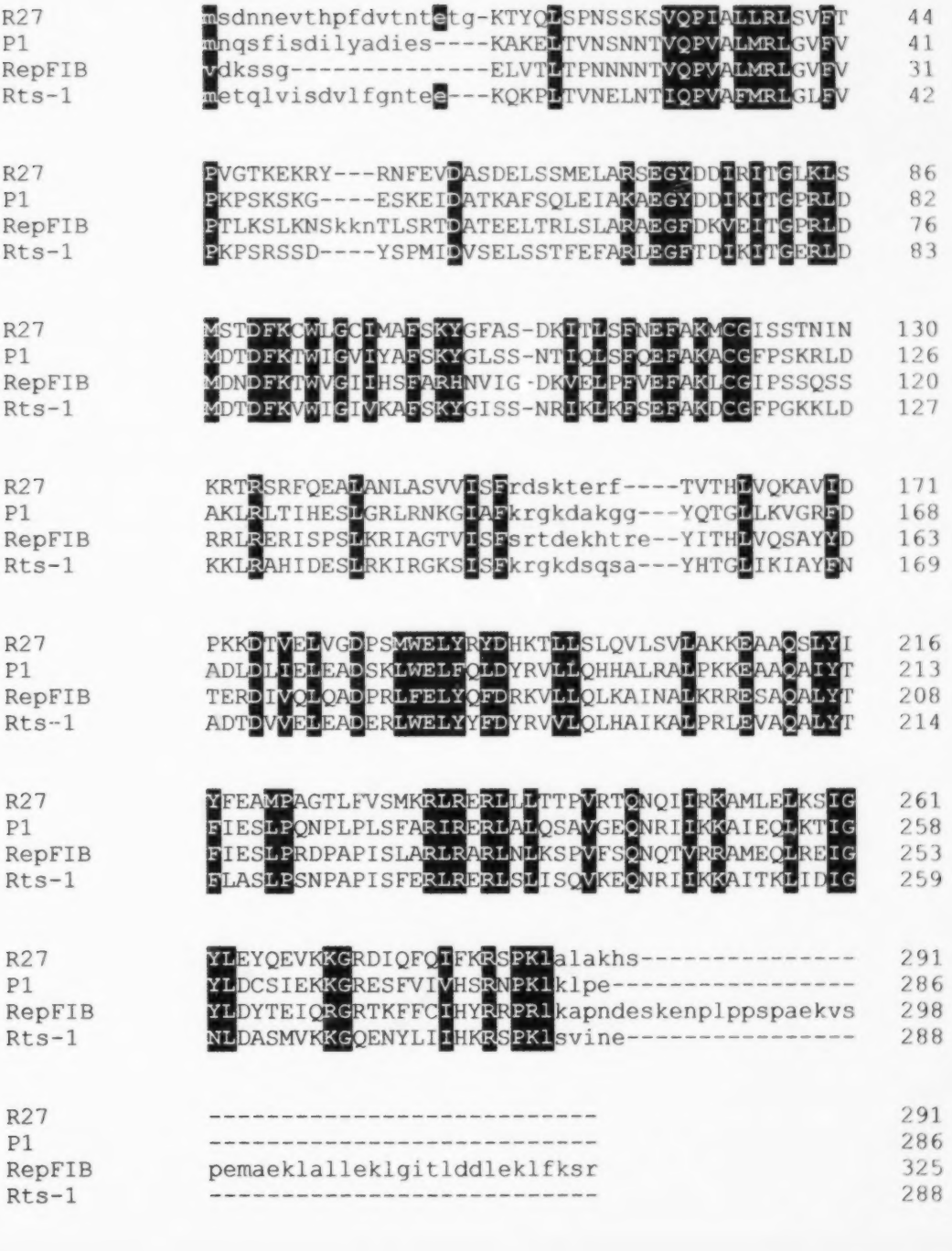
[c]β-galactosidase activity was determined by the method of Miller (91). Each strain was assayed in triplicate and the results averaged.

H. Homology with Rep proteins from other plasmids.

Computer databank searches revealed homology of the Rep protein encoded by the large ORF present in the R27 replicon with the replication initiation proteins of plasmids from three different incompatibility groups. These include RepA from P1 (IncY) (1), RepA from Rts-1 (IncT) (66), and the replication initiator encoded by RepFIB, present on numerous IncFI plasmids, including P307 (124), pColV-K30 (111), and F (73). The replication proteins encoded by these plasmids are relatively conserved, having similar molecular weights (32 - 37 kDa), comparable predicted pI's (9.7 - 10.1), and 37 - 42% amino acid identity at the sequence level. As well, the organization of the basic replicon is similar in all cases. Repeated iterons surround the ORF encoding the replication protein, and origin elements exist upstream of the ORF (DnaA boxes, *dam*-methylase sites). The alignment of the three replication proteins, as well as the protein encoded by the R27 basic replicon, is shown in Figure III-7.

Figure III-7 Alignment of replication proteins.

Protein alignments were generated with MACAW (Multiple Alignment Construction and Analysis Workbench, 130). Aligned residues (blocks) are shown in upper case characters. Shading is based on the mean of all pairwise similarity scores for a particular column (PAM120 similarity matrix) - the darker the shade, the higher the average similarity score. Proteins aligned are as follows: R27, putative replication protein encoded by the minimal replicon of R27; P1, RepA protein from minimal replicon of P1; Rts-1, replication protein from mini-Rts-1; REP FIB, replication protein encoded by RepFIB replicon of P307.


R27 msdnnevthpfdvtntetg-KTYQLSPNSSKSVQPIALLRLSVFT 44
P1 mnqsfisdilyadies----KAKELTVNSNNTVQPVALMRLGVFV 41
RepFIB vdkssg----------ELVTLTPNNNNTVQPVALMRLGVFV 31
Rts-1 metqlvisdvlfgntee---KQKPLTVNELNTIQPVAFMRLGLFV 42
R27 PVGTKEKRY---RNFEVDASDELSSMELARSEGYDDIRITGLKLS 86
P1 PKPSKSKG----ESKEIDATKAFSQLEIAKAEGYDDIKITGPRLD 82
RepFIB PTLKSLKNSkknTLSRTDATEELTRLSLARAEGFDKVEITGPRLD 76
Rts-1 PKPSRSSD----YSPMIDVSELSSTFEFARLEGFTDIKITGERLD 83
R27 MSTDFKCWLGCIMAFSKYGFAS-DKITLSFNEFAKMCGISSTNIN 130
P1 MDTDFKTWIGVIYAFSKYGLSS-NTIQLSFQEFAKACGFPSKRLD 126
RepFIB MDNDFKTWVGIIHSFARHNVIG-DKVELPFVEFAKLCGIPSSQSS 120
Rts-1 MDTDFKVWIGIVKAFSKYGISS-NRIKLKFSEFAKDCGFPGKKLD 127
R27 KRTRSRFQEALANLASVVISFrdskterf----TVTHLVQKAVID 171
P1 AKLRLTIHESLGRLRNKGIAFkrgkdakgg---YQTGLLKVGRFD 168
RepFIB RRLRERISPSLKRIAGTVISFsrtdekhtre--YITHLVQSAYYD 163
Rts-1 KKLRAHIDESLRKIRGKSISFkrgkdsqsa---YHTGLIKIAYFN 169
R27 PKKDTVELVGDPSMWELYRYDHKTLLSLQVLSVLAKKEAAQSLYI 216
P1 ADLDLIELEADSKLWELFQLDYRVLLQHHALRALPKKEAAQAIYT 213
RepFIB TERDIVQLQADPRLFELYQFDRKVLLQLKAINALKRRESAQALYT 208
Rts-1 ADTDVVELEADERLWELYYFDYRVVLQLHAIKALPRLEVAQALYT 214
R27 YFEAMPAGTLFVSMKRLRERLLLTTPVRTQNQIIRKAMLELKSIG 261
P1 FIESLPQNPLPLSFARIRERLALQSAVGEQNRIIKKAIEQLKTIG 258
RepFIB FIESLPRDPAPISLARLRARLNLKSPVFSQNQTVRRAMEQLREIG 253
Rts-1 FLASLPSNPAPISFERLRERLSLISQVKEQNRIIKKAITKLIDIG 259
R27 YLEYQEVKKGRDIQFQIFKRSPKlalakhs---------- 291
P1 YLDCSIEKKGRESFVIVHSRNPKlklpe---------- 286
RepFIB YLDYTEIQRGRTKFFCIHYRRPRlkapndeskenplppspaekvs 298
Rts-1 NLDASMVKKGQENYLIIHKRSPKlsvine---------- 288
R27 ---------- 291
P1 ---------- 286
RepFIB pemaeklalleklgitlddleklfksr 325
Rts-1 ---------- 288

I. Copy number and stability of REP constructs

Copy number of a series of replicon-containing subclones was determined and compared to that of the parent plasmid, R27. Copy number was determined by dot blot analysis of total DNA isolated from strains harboring R27 and its replicon-containing derivatives. Since IncH plasmids have a known copy number of one to two per chromosome equivalent (146), R27 was used as a standard for comparison in the experiment. Copy numbers were determined using *E. coli* SF800 as the host, in which replication from ColE1 replicons (present in pUC vectors) is inhibited. Deletion of all downstream iterons in the minimal replicon results in an approximate 8-fold increase in copy number compared to that of R27.

In order to ascertain the presence of determinants contributing to plasmid maintenance, several replication proficient subclones were examined for their stability in the absence of antibiotic selection. In all cases, the *polA* strain SF800 was used as the host to eliminate any contribution from the ColE1 replicon of the vector in those subclones containing pUC sequences. Near the end of the experiment (70 - 90 generations of non-selective growth), a sample of the culture was examined to ensure reversion of the PolA phenotype had not occurred, by testing its ability to transform to kanamycin resistance with the ColE1-based plasmid pKIXX. In all cases, the culture maintained its PolI$^-$ phenotype, as evidenced by its inability to be

transformed with pKIXX DNA. Neither pDT2123, nor any Rep$^+$ constructs derived from pDT2123 proved to be stable, with loss rates of 2-3% per generation.

J. Functional analysis of the R27 minimal replicon

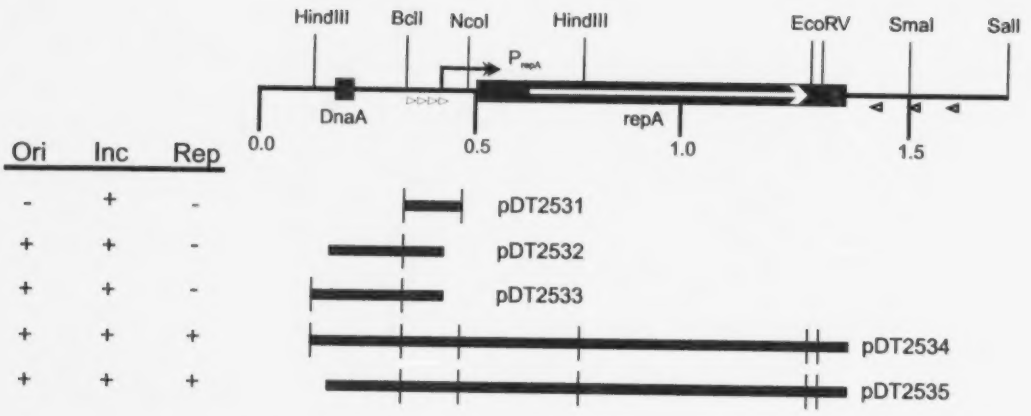
Dissection of the functional domains of the minimal replicon was undertaken by cloning regions of the replicon to pUCBM20. The subclones derived from pDT2123 are depicted in Figure III-8. Plasmids pDT2532, pDT2533, pDT2534, and pDT2535 were constructed by amplifying the appropriate regions of pDT2123 by PCR. Each subclone was tested for three activities. Incompatibility was tested as described above. The construct was deemed replication-proficient (Rep$^+$) if it could transform *E. coli* strain SF800 (*polA*) to ampicillin resistance. The presence of a functional origin of replication was examined by transforming each subclone into *E. coli* strain SF800 which carried plasmid pDT2376 (Km^R). pDT2376 supplies the *trans*-acting components necessary to initiate replication at a functional origin. The subclone was deemed Ori$^+$ if Ap- and Km-resistant colonies could be selected following the transformation. In all cases where colonies appeared under double selection, plasmid DNA was examined from several isolated colonies by agarose gel electrophoresis to ensure that a recombinational event had not occurred.

pDT2531, which extends from the B*cl*I site to the N*co*I site, contains the four upstream iterons, and the promoter for the *repA* gene. Neither the DnaA binding sites, nor the Dam methylase recognition sequences are present in this clone. As shown in Figure III-8, this construct expresses incompatibility with the minimal replicon, but it is not replication-proficient nor does it harbour a functional replication origin. Plasmids pDT2532 and pDT2533 carry all of the upstream iterons, the Dam methylase recognition sites and the DnaA binding sites. Both subclones express incompatibility and possess a functional origin of replication, but are incapable of autonomous replication in the absence of PolI. Plasmids pDT2534 and pDT2535 contain all of the upstream elements mentioned as well as the coding sequence for RepA, but none of the downstream iterons. These subclones are replication-proficient and exhibit incompatibility.

These data show that R27 possesses a PolI-independent replicon whose structure and replicative properties place it among a large group of related iteron-controlled plasmids.

Figure III-8 Subcloning and functional analysis of the minimal replicon.

The sequenced region of the minimal replicon is depicted with relevant restriction sites indicated. The RepA open reading frame and putative DnaA binding sites are indicated in the map, and are based on the DNA sequence (see Figure III-3). The large arrow within the RepA orf indicates its orientation. Seven small arrowheads represent 19-bp iterons; the direction of the arrowhead indicates the orientation of the iteron. In addition, the approximate location of the RepA promoter is indicated. Shown below the map are a number of subclones, constructed as described in the text. Each subclone was tested for its ability to replicate in a *polA* host (Rep), to mediate incompatibility (Inc), and to replicate in a *polA* host in the presence of pDT2376 (Ori).


HindIII
BclI
NcoI
HindIII
EcoRV
SmaI
SalI
P_{repA}
DnaA
repA
0.0
0.5
1.0
1.5
Ori
Inc
Rep
- + -
+ + -
+ + -
+ + +
+ + +
pDT2531
pDT2532
pDT2533
pDT2534
pDT2535

Chapter IV Other maintenance elements of R27[2]

A. RepHI1B and the IncHI1 determinant

A fragment was previously isolated from the IncHI1 plasmid TR6 and shown to hybridize specifically to all IncHI1 plasmids but not to plasmids of the IncHI2 group (29). Despite the lack of homology between this fragment and plasmids of the HI2 group, it expresses incompatibility with plasmids of both the IncHI1 and IncHI2 groups. This fragment has been sequenced (46) and the locus imparting incompatibility designated IncHI1. In order to map the location of this determinant on R27, an *Eco*RI-*Hin*dIII fragment, encompassing the *inc* determinant, was excised from pULB2436 (a subclone of TR6 carrying the *inc* determinant) and radiolabelled by nick translation for use as a probe in hybridization studies. This probe was hybridized with restriction fragments of R27 blotted to nitrocellulose. The hybridization revealed that DNA with homology to the probe exists within the *Sal*I-F fragment of R27. The precise location of the homologous DNA was determined by probing restriction digests of pDT1233, a cosmid clone of R27 that contains the *Sal*I-F fragment (Figure IV-1).

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[2] Portions of this chapter have been published. **Newnham, P.J., and Taylor, D. E.** 1990. Plasmid 23:107-118. **Gabant, P., Newnham, P., Taylor, D., and Couturier, M.** 1993. J. Bacteriol. 175:7697-7701.

A second PolI-independent replicon, RepHI1B, was cloned and characterized by Gabant *et al.* (45). This replicon was originally cloned from the IncHI1 plasmid pIP522. Nucleotide sequence analysis revealed this replicon to be distantly related to RepHI1A, with significant homology in the encoded replication initiator proteins, but to possess no homology at the DNA level. The RepHI1B replicon was located on the R27 map by Southern hybridization. A fragment encompassing a region of the RepHI1B replicon was radiolabelled by nick translation and used as a probe with restriction fragments of R27 blotted to nitrocellulose. The hybridization revealed that DNA with homology to the probe exists within the S*al*I-E and S*al*I-I fragments of R27. Fine mapping demonstrated that the RepHI1B replicon straddles the S*al*I site between S*al*I fragments E and I (see Figure IV-1 and Figure IV-2).

A series of fragments, subcloned from pDT1233, was tested for expression of incompatibility towards R27. In addition, each cloned fragment was tested for its ability to transform a *polA E. coli* host (SF800), indicating the presence of a PolI-independent replicon within the cloned fragment. These results are summarized in Figure IV-1.

Clones containing the IncHI1 locus but not the RepHI1A replicon (pDT2113, pDT2117, pULB2436, pULB2447) are not autoreplicative, which suggests that this region is not directly involved in plasmid replication. These clones do however, express incompatibility with R27, as expected. It is

also interesting to note that while neither RepHI1A nor RepHI1B expresses incompatibility with R27 on its own, subclone pDT2354, which contains a complete copy of RepHI1A and a partial copy of RepHI1B, expresses strong incompatibility with R27. The location of each of these determinants on the R27 circular map is indicated in Figure IV-2.

Figure IV-1 Subcloning and hybridization analysis of pDT1233

Restriction map of the region of R27 cloned in pDT1233. Restriction sites are as follows: *Eco*RI (E), *Sal*I (S), *Xba*I (X). This clone contains the *Sal*I-E and the *Sal*I-F fragments of R27. The location of the two replicons RepHI1A and RepHI1B are represented by boxes above the restriction map. IncHI1 is the incompatibility determinant. Subclones of this region are represented beneath the restriction map; the shading of these boxes reflects their ability to express incompatibility (Inc) or to replicate in a *polA* host (Rep). pDT2113, pDT2117, pULB2436, and pULB2447 (dark shading) all express incompatibility against IncHI1 plasmids, but do not replicate in a polA host. pDT2120, pDT2123, and pDT2130 (light shading) replicate in a *polA* host, but do not express incompatibility against R27. pDT2354 (black) is capable of replicating in a *polA* host and also expresses incompatibility against R27. Unshaded constructs neither express incompatibility nor replicate in the absence of PolI. The RepHI1B replicon maps at the border of pDT1233, as shown, and thus only a non-functional portion of RepHI1B is present in this clone.

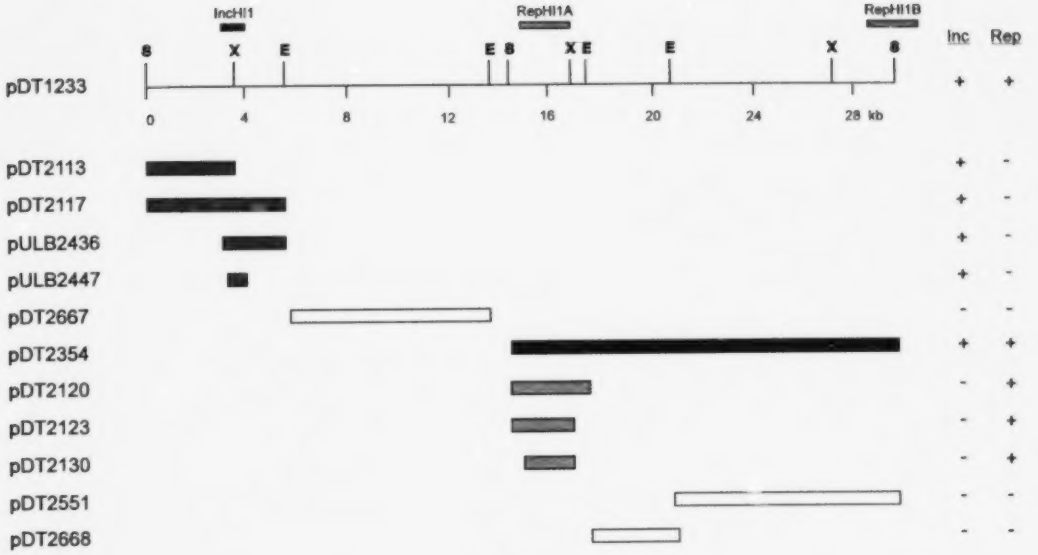
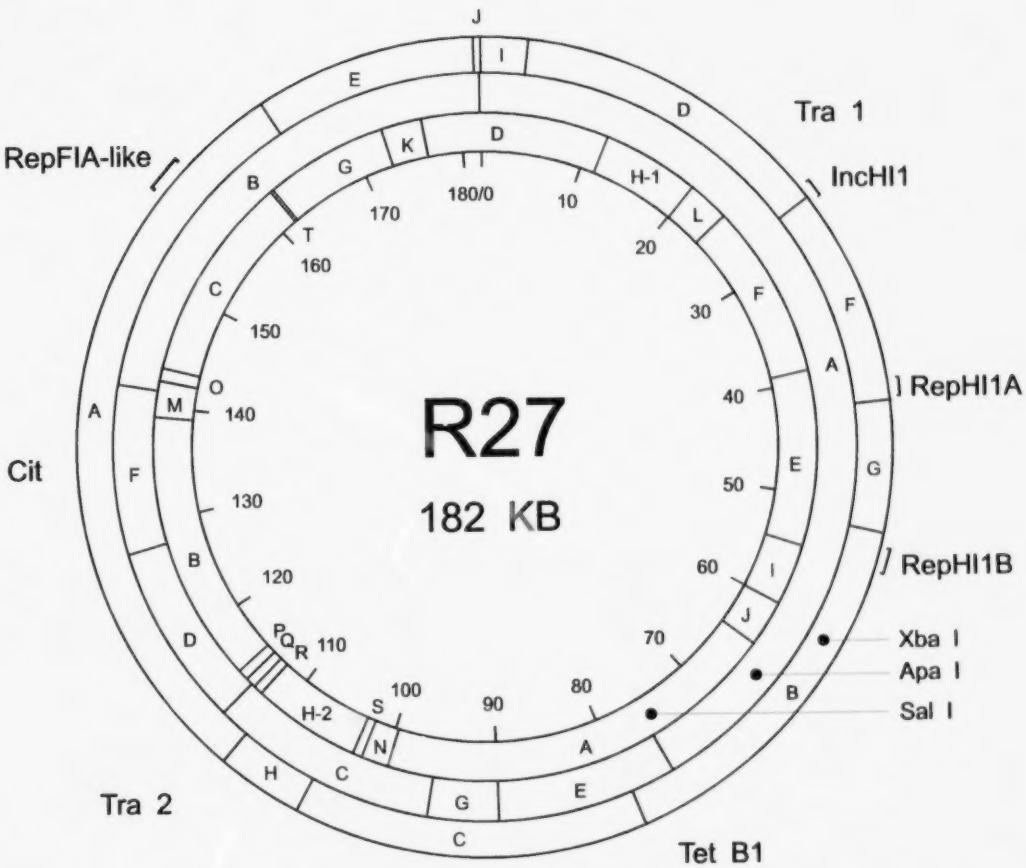

IncHI1
RepHI1A
RepHI1B
S
X
E
E
S
X
E
E
X
S
Inc
Rep
pDT1233
0
4
8
12
16
20
24
28 kb
pDT2113
pDT2117
pULB2436
pULB2447
pDT2667
pDT2354
pDT2120
pDT2123
pDT2130
pDT2551
pDT2668

Figure IV-2 Location of maintenance elements specific for IncHI1 plasmids on the R27 map

The plasmid is divided into regions according to the original R27 map and the nomenclature used in this thesis. The three functional replicons, RepFIA-like, RepHI1A, and RepHI1B, are indicated. The incompatibility determinant is indicated as IncHI1.

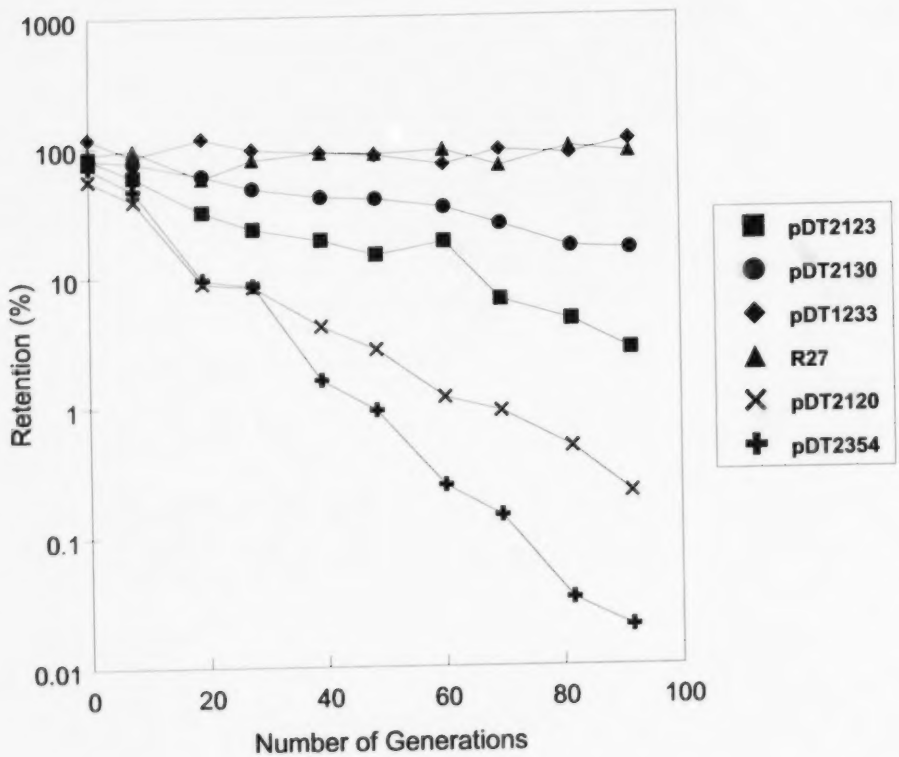

R27
182 KB
RepFIA-like
Tra 1
IncHI1
RepHI1A
RepHI1B
Cit
Tra 2
Tet B1
Xba I
Apa I
Sal I
180/0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170

B. Stability

The stability of several of the replication-proficient subclones of pDT1233 was tested. Stability was examined in the *polA* host SF800, so that the only replicon contributing to the replication of the clone was the RepHI1A replicon present in the cloned fragment. The results of this analysis are depicted in Figure IV-3. Plasmid pDT1233, which harbours both the RepHI1A replicon and the IncHI1 locus, was observed to be stable when transformed into a *polA* strain, which causes the plasmid to initiate replication solely from the RepHI1A origin. Subclones containing only the RepHI1A replicon, without the Inc locus are unstable in the same background (Figure IV-3).

Figure IV-3 Stability of R27 and subclones in a *polA* background

In this background, replication is mediated solely by the presence of PolI-independent replicons within the testes plasmids. The vector replicon does not function. Stability was tested as follows: Overnight cultures, grown in the presence of selective antibiotic, were diluted 1:1000 in fresh media (Luria broth) and allowed to grow at 37°C in a shaking incubator for an additional 7 to 15 hours. Additional 1:1000 dilutions of the culture were made at 7 to 15 hour intervals for the duration of the experiment. Prior to each dilution step, a sample of the culture was plated on selective and non-selective media (Luria broth, 1.5% agar) to determine % retention of the plasmid. The x-axis indicates the proportion of the culture maintaining plasmid-borne resistance (ampicillin resistance) at any given time point. The y-axis indicates the number of generations of growth. Plasmids tested are indicated in the legend.


1000
100
10
1
0.1
0.01
Retention (%)
0
20
40
60
80
100
Number of Generations
pDT2123
pDT2130
pDT1233
R27
pDT2120
pDT2354

C. Pervasiveness of the RepHI1A and RepHI1B replicons within the H complex

Hybridization experiments were carried out to determine if the RepHI1A and RepHI1B replicons are specific to the HI1 incompatibility subgroup. A collection of 260 strains containing plasmids representative of numerous different incompatibility groups (IncB, IncC, IncD, IncFI, IncFII, IncFIII, IncFIV, IncFme, IncFV, IncFVI, IncFVII, IncHI1, IncHI2, IncHII, IncI1, IncI2, Incγ, IncJ, IncK, IncM, IncN, IncP, IncQ, IncT, IncU, IncV, IncW, IncX, IncY and IncZ) was used to screen for the presence of the two IncHI1 replicons. Colony blots were prepared as described (29). Both replicons hybridized to all represented IncHI1 plasmids and failed to hybridize with plasmids from any other incompatibility group.

Chapter V Characteristics of the transfer system of R27

A. Complementation of R27 Tra⁻ mutants with pHH1508a

Plasmids of both H incompatibility groups (IncHI and IncHII) encode sex pili that are morphologically and serologically related. Although plasmids of each incompatibility group possess no detectable homology at the DNA level, the observed similarity in conjugative pili may extend to other components of their respective transfer systems, such that plasmids of one incompatibility group may be capable of complementing defects in the transfer apparatus of the other group. In order to test this hypothesis, a series of complementation studies were undertaken using the wild type IncHII plasmid pHH1508a to complement transfer deficient insertion mutants of the IncHI1 plasmid R27.

Complementation of R27 Tra⁻ mutants by pHH1508a was tested in four different *E. coli* host strains at 26°C (Table V-1) and 37°C (Table V-2). R27 Tra- mutants employed in this study were generated by transposon insertion mutagenesis using either Tn5 or Tn7 (144, 152). Insertion locations are depicted in Figure I-1. The most efficient host for complementation was *E. coli* J53-1. The wild type R27 transferred with a 10-fold higher frequency at both 26°C and 37°C from *E. coli* J53-1(R27, pHH1508a) than from *E. coli* J53-1(R27). The Tra⁻ mutants of R27 fell broadly into two groups based on mating frequencies at 26°C and 37°C from J53-1(R27 Tra⁻, pHH1508a). One

group (pDT916, pDT917, pDT921, pDT922 and pDT932) transferred with moderate frequency ($2.0x10^{-6}$ - $6.6x10^{-4}$ transconjugants/recipient) at 26°C, while at 37°C higher transfer frequencies were noted ($1.1x10^{-3}$ - $7.3x10^{-2}$). The second group of plasmids (pDT1015, pDT1016, pDT1018, pDT1030, pDT1033) exhibited lower mating frequencies at both temperatures ($4.2x10^{-8}$ - $1.3x10^{-5}$). Comparisons of transfer frequencies at 37°C and 26°C (Table V-3) demonstrated that, whereas R27 alone or in combination with pHH1508a transferred in excess of 5000 fold better at 26°C than 37°C, transfer frequencies of mutants were from 1.3 to 900 fold greater at 37°C than at 26°C.

Complementation testing was also carried out using three other hosts, to determine if host genotype could affect the complementation process. Since J53-1 is a recombinant proficient host ($RecA^+$), two $RecA^-$ and one additional $RecA^+$ hosts were tested (Table V-1 and Table V-2). Due to the large size of the R27 Tra^- mutants (>180kb), transformation of the various strains with these plasmids was very inefficient. Certain hosts were extremely difficult to transform, and for this reason, not all Tra^- mutants have been tested in all hosts.

Frequencies of transfer in other hosts varied but were, in general, considerably lower than those seen in J53-1. For purposes of comparison, the mating frequency of the wild type plasmid R27 was initially compared. At 26°C, R27 transferred with frequencies almost identical to those seen from

J53-1. However, in the presence of pHH1508a, R27 transferred at a much lower frequency from JE2571 ($4.0x10^{-7}$ transconjugants/recipient) and failed to transfer at all from HB101. No transfer was observed in either host at 37°C, regardless of the presence or absence of pHH1508a. R27 did transfer from JC1569 at 37°C when in the presence of pHH1508a, with frequencies similar to those observed with J53-1 as the host. Unlike the case in which J53-1 was used as the host, transfer frequencies of the R27 Tra^- mutants at 37°C were approximately equal to or lower than those at 26°C when any of HB101, JC1569, or JE2571 were used as hosts for complementation.

Several transconjugants from selected matings were tested to determine if the two co-resident plasmids had recombined as a means of transfer. Transconjugants possessing antibiotic resistance markers for both the HI and HII plasmids (e.g. Tc^R,Te^R) were mated to appropriate recipients by means of the standard broth mating protocol employed in this study. Transconjugants resulting from this cross were tested for the presence of one or both plasmids by transferring to media selective for each plasmid. All techniques employed in this study, including monitoring for separate transfer of coresident plasmids and restriction endonuclease analysis of transconjugant plasmids, indicated that in most instances (>95% of those analyzed), the coresident plasmids had not undergone recombination prior to or subsequent to transfer.

These data demonstrate that deficiencies in the R27 transfer apparatus can be complemented by the presence of a plasmid of the IncHII group, and that IncHI1 transfer thermosensitivity can also be similarly alleviated .

Table V-1 Complementation between R27 Tra⁻ mutants and pHH1508a at 26°C

	Mating Frequencies[a]			
R27 Tra⁻[(b)]	J53-1	HB101	JC1569	JE2571
pDT916	2.0×10^{-6}	8.6×10^{-8}	5.2×10^{-7}	N.D.[d]
pDT917	2.0×10^{-6}	$<10^{-8}$	2.7×10^{-8}	N.D.
pDT921	6.6×10^{-4}	$<10^{-8}$	N.D.	N.D.
pDT922	5.9×10^{-5}	7.6×10^{-8}	1.8×10^{-8}	N.D.
pDT932	1.6×10^{-4}	3.5×10^{-8}	1.3×10^{-8}	N.D.
pDT1015	9.5×10^{-8}	3.7×10^{-7}	N.D.	9.4×10^{-7}
pDT1016	1.2×10^{-6}	9.7×10^{-8}	N.D.	5.8×10^{-6}
pDT1018	4.2×10^{-8}	N.D.	N.D.	2.3×10^{-6}
pDT1030	1.0×10^{-5}	1.9×10^{-7}	N.D.	2.9×10^{-7}
pDT1033	1.6×10^{-7}	6.7×10^{-8}	N.D.	9.8×10^{-7}
R27[c] (alone)	6.0×10^{-4}	1.5×10^{-4}	1.3×10^{-3}	1.3×10^{-3}
R27[d] (+pHH1508a)	7.0×10^{-3}	$<10^{-8}$	1.0×10^{-4}	4.0×10^{-7}

[a]Mating frequencies determined as transconjugants per recipient in overnight mating experiments. Each transfer experiment was repeated four times; average frequency reported

[b]Insertion sites for Tra⁻ mutants are indicated in Figure I-1

[c]Mating frequency determined in absence of pHH1508a

[d]Mating frequency determined in presence of pHH1508a

[e]N.D., not determined

Table V-2 Complementation between R27 Tra⁻ mutants and pHH1508a at 37°C

	Mating Frequencies[a]			
R27 Tra⁻[(b)]	J53-1	HB101	JC1569	JE2571
pDT916	1.1×10^{-3}	$<10^{-8}$	$<10^{-8}$	N.D.[d]
pDT917	1.8×10^{-3}	$<10^{-8}$	2.0×10^{-8}	N.D.
pDT921	7.3×10^{-2}	$<10^{-8}$	N.D.	N.D.
pDT922	1.7×10^{-3}	$<10^{-8}$	5.9×10^{-8}	N.D.
pDT932	1.5×10^{-3}	$<10^{-8}$	2.8×10^{-7}	N.D.
pDT1015	5.2×10^{-6}	$<10^{-8}$	N.D.	$<10^{-8}$
pDT1016	7.1×10^{-6}	$<10^{-8}$	N.D.	5.0×10^{-8}
pDT1018	9.2×10^{-7}	N.D.	N.D.	6.0×10^{-8}
pDT1030	1.3×10^{-5}	$<10^{-8}$	N.D.	1.0×10^{-8}
pDT1033	6.7×10^{-6}	$<10^{-8}$	N.D.	$<10^{-8}$
R27[c] (alone)	$<10^{-8}$	$<10^{-8}$	$<10^{-8}$	$<10^{-8}$
R27[d] (+pHH1508a)	1.3×10^{-7}	$<10^{-8}$	1.7×10^{-7}	$<10^{-8}$

[a]Mating frequencies determined as transconjugants per recipient in overnight mating experiments Transfer experiments repeated four times; average frequency reported

[b]Insertion sites for Tra⁻ mutants are indicated in Figure I-1

[c]Mating frequency determined in absence of pHH1508a

[d]Mating frequency determined in presence of pHH1508a

[e]N.D., not determined

Table V-3 Comparison of transfer complementation from *E. coli* J53-1(R27, pHH1508a) at 26°C and 37°C

		Transfer[a] at		
Plasmid	Tra Region	26°C	37°C	37°C/26°C[b]
pDT916	Tra1	2.0×10^{-6}	1.1×10^{-3}	550
pDT917	Tra1	2.0×10^{-6}	1.8×10^{-3}	900
pDT922	Tra1	5.9×10^{-5}	1.7×10^{-3}	28
pDT1016	Tra1	1.2×10^{-6}	7.1×10^{-6}	5.9
pDT1018	Tra1	4.2×10^{-8}	9.2×10^{-7}	21
pDT921	Tra2	6.6×10^{-4}	7.3×10^{-2}	100
pDT932	Tra2	1.6×10^{-4}	1.5×10^{-3}	9
pDT1033	Tra2	1.6×10^{-7}	6.7×10^{-6}	42
pDT1015	Tra1+Tra2	9.5×10^{-8}	5.2×10^{-6}	55
pDT1030	Tra1+Tra2	1.1×10^{-5}	1.3×10^{-5}	1.3
				26°C/37°C[c]
R27 alone		6.0×10^{-4}	$<1 \times 10^{-8}$	>6000
R27 + pHH1508a		7.0×10^{-3}	1.3×10^{-7}	5400

[a]Mating frequencies reported as transconjugants per recipient

[b]Ratio of transfer at 37°C to transfer at 26°C

[c]Ratio of transfer at 26°C to transfer at 37°C

B. Complementation of R27 Tra⁻ mutants with cosmid clones of R27

Cosmids containing cloned fragments of R27 encompassing the region of transposon insertion for each Tra⁻ plasmid were selected for testing complementation. Because of the incompatibility expressed by cosmid clones containing the *Sal*I-F fragment of R27 towards a co-resident IncHI1 plasmid, many of the Tra⁻ mutants with insertions in the Tra1 region could not be tested in this manner. Mating frequencies for the Tra⁻ plasmids, when complemented with appropriate cosmids, are listed in Table V-4. Complementation *in trans* was demonstrated by the transfer of the Tra⁻ plasmid at normal or near-normal frequencies (10^{-4} transconjugants/ recipient). Of seven Tra⁻ mutants tested with insertions in the Tra 2 region, four were complemented *in trans* (pDT921, pDT932, pDT1023, pDT1033). The other mutants in Tra 2 remained transfer deficient, or transferred only at very low frequencies ($<7.0 \times 10^{-8}$) in the presence of an R27 cosmid, as expected since no clone was available which contained regions of R27 corresponding to the insertion sites of these mutants. Three Tra⁻ mutants with insertions into the Tra 1 region were tested in complementation experiments with cosmid pDT1689. Of these, one mutant (pDT1185) was able to transfer at approximately 10^{-4} transconjugants/recipient, whereas the others, (pDT1180 and pDT987) did not transfer or transferred at very low frequency ($<4 \times 10^{-8}$ transconjugants/ recipient).

Table V-4 Complementation between R27 tra⁻ and R27 cosmid clones

R27 Tra⁻	Cosmid #[a]	Mating Frequency[b]	Tra Region[c] Involved
pDT1180	pDT1689	$<1.0 \times 10^{-8}$	Tra1
pDT1185	pDT1689	9.8×10^{-5}	Tra1
pDT987	pDT1689	3.0×10^{-8}	Tra1
pDT987	pDT1690	$<1.0 \times 10^{-8}$	Tra1
pDT921	pDT1692	9.2×10^{-4}	Tra2
pDT932	pDT1692	3.9×10^{-4}	Tra2
pDT1023	pDT1692	5.2×10^{-4}	Tra2
pDT1033	pDT1692	2.9×10^{-3}	Tra2
pDT978	pDT1692	$<1.0 \times 10^{-8}$	Tra2
pDT982	pDT1692	6.7×10^{-8}	Tra2
pDT988	pDT1692	4.2×10^{-8}	Tra2

[a]Regions of R27 cloned into cosmids are shown in Figure I-2

[b]Broth matings carried out at 26°C. Mating frequency determined as transconjugants/recipient

[c]Tra regions of R27 are indicated in Figure I-1

C. Temperature effects on R27 transfer

A number of experiments were devised to investigate the effects of sudden temperature shifts on transfer efficiency of R27. Previous data have demonstrated that the growth temperature of the donor culture is the critical element in determining transfer frequency; the incubation temperature of the recipient has little effect on transfer frequency (147). The studies reported herein were designed to investigate in more detail the effect of temperature on donor efficiency.

Since the wild-type plasmid transfers with relatively low frequency (~1.0×10^{-6} transconjugants per donor in 2 hour matings, ~1.0×10^{-2} transconjugants per recipient in overnight matings), a derepressed derivative of R27 (pDT1942) was employed in this study. *E. coli* strain JE2571-2 was used as the host organism and strain J53-1 was used as the recipient in all of these experiments. Matings were performed in LB, as described. Matings involving a temperature shift were carried out in thin-walled tubes using small culture volumes. Under these conditions, temperature equilibration occurred in less than 1 minute.

No transfer was detectable in one hour matings, when donor and recipient were preincubated at 37°C and the mating performed at the same temperature. If preincubation and transfer were carried out at 26°C, transconjugants were detected at a rate of approximately 1.0×10^{-3} to 3.0 x

10^{-3} per input donor cell. When the donor culture was preincubated at 37°C, and the conjugation carried out at 26°C, transconjugants were not detectable for at least 100 min. after mixing donor and recipient cultures.

In another experiment, the donor culture was incubated at 37°C for short periods of time, immediately before mixing with the recipient culture (pre-incubated at 26°C), and the transfer carried out for one hour at 26°C. The results of this experiment are detailed in Table V-5. Exposures to the inhibitory temperature as brief as 5 min. had a dramatic impact on the conjugational proficiency of the donor culture. Exposures longer than 20 min. completely abolished detectable transfer in one hour matings.

Table V-5 Effect of high temperature exposure on donor efficiency

Elapsed Time at 37°C[a] (minutes)	Mating Frequency[b] (transconjugants/donor)
0	1.7×10^{-3}
5	6.9×10^{-4}
10	2.7×10^{-4}
15	5.3×10^{-5}
20	1.1×10^{-5}
25	$<1 \times 10^{-7}$

[a]Denotes elapsed time the donor culture was incubated at 37°C immediately before mating at 26°C.

[b]Mating frequency determined after 1 hour conjugation at 26°C.

The effect of transient temperature shifts to 37°C during the mating was investigated in another experiment. In this case, pre-incubation of both donor and recipient cultures was carried out at 26°C. The cultures were mixed together, and at various time points, the mating mixture was shifted to 37° for either 1 min. or 5 min. The mating was continued at 26°C and transfer frequency determined. The effect of the transient temperature shift on one hour transfer efficiency is shown in Figure V-1. In every case, brief shifts to 37°C during mating reduced the efficiency of the transfer. A 5 min. pulse reduced transfer frequency by 3- to 6-fold, when introduced any time during the first 30 min. of the mating.

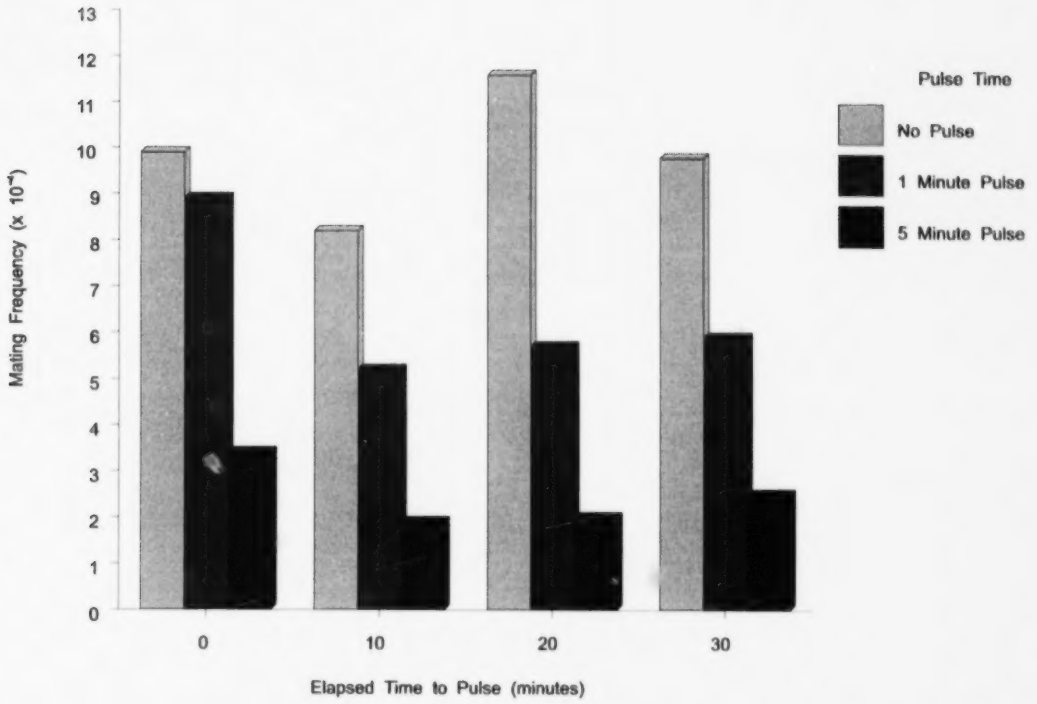


Figure V-1 Transient temperature shifts during transfer

Mating cultures were briefly shifted to 37°C at various time points in a one hour conjugation, as indicated on the X-axis. Incubation time at the elevated temperature is indicated by bar color, as shown in the legend. Transfer frequencies are plotted and expressed in transconjugants per donor cell. In each instance, the donor used in the experiment was J53-1(pDT1942) and the recipient was RG192.

Finally, an experiment was carried out to compare the effect of shifting temperature during conjugation to that of physically disrupting mating during the conjugation. In this experiment, donor and recipient cultures were pre-incubated at 26°C. The cultures were mixed together and mating initiated at 26°C. At various time points thereafter, samples of the mating mixture were either shifted to 37°C, or vortexed for 15 sec. and placed on ice. Media temperature equilibrated in less than 45 sec. under the conditions employed in this experiment. Incubation was continued until one hour had elapsed from the time the mating began. Transfer frequencies were determined for each sample. The results of this experiment are depicted in Figure V-2.

When conjugation was physically disrupted, no transfer could be detected in the first 10 min. Transconjugants began to appear at 15 min. after mating initiation. The number of transconjugants in the population rapidly increased from that point and began to level off after 35-40 min. If the temperature of the mating culture was shifted to 37°C within the first five minutes of the conjugation, no transconjugants appeared in one hour matings. Temperature shifts later in the conjugation permitted the appearance of transconjugants at reduced levels.

These data demonstrate that exposure to higher temperatures produces a rapid and lasting inhibition of plasmid transfer.

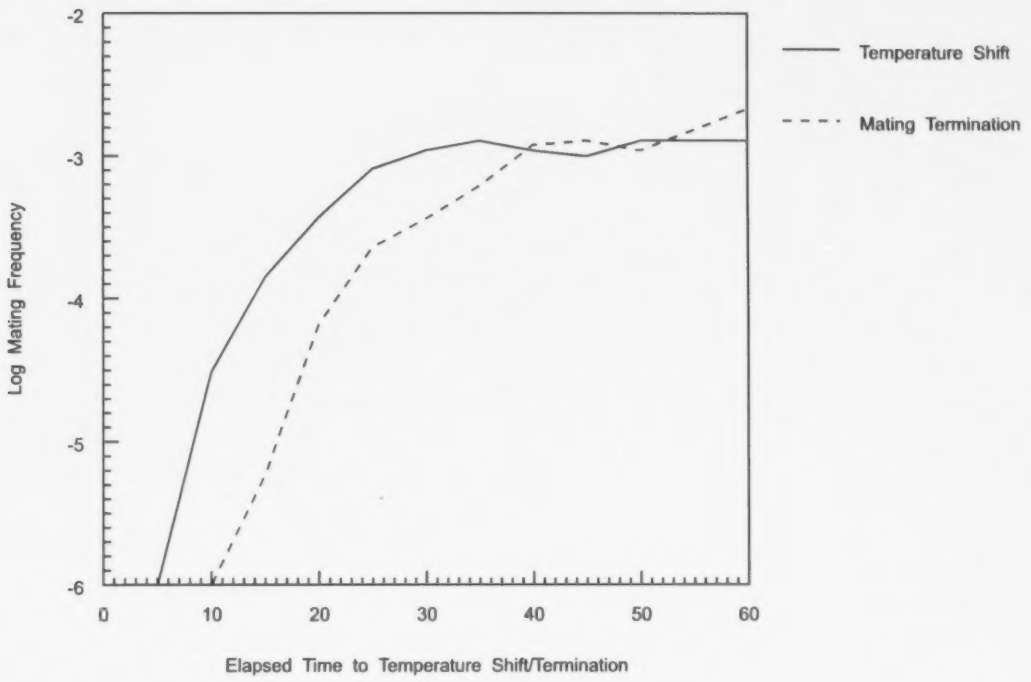


Figure V-2 Comparison of temperature shifts and physical interruption during transfer of R27

Mating cultures were either shifted to 37°C or vortexed and placed on ice at various time points in a one hour conjugation, as indicated on the X-axis. The mating frequency is plotted on the Y-axis, and expressed as transconjugants per donor cell.

Chapter VI Characteristics of the conjugative pili encoded by R27

A. Isolation of conjugative pili

In order to undertake a detailed analysis of the conjugative pili encoded by R27, quantities of purified pili were initially isolated. Since the wild-type plasmid is naturally repressed for transfer, a relatively low percentage of cells harboring R27 express the conjugative pili encoded by the plasmid (less than 1 in 1000 cells). Because of this, a derepressed derivative of R27, pDT1942 (81), was used as a source of the pili. This derivative carries a transposon insertion (TnLacZ) in the transfer repressor gene carried on R27 (*htd*A - located in fragment S*al*I-H1), resulting in dramatically higher transfer frequency and concomitant pilus expression (166). To eliminate contamination from other pili and flagella produced by the host bacteria, a "bald" strain of *E. coli* (JE2571) was used as the host for this experiment. This strain produces neither common (Type 1) pili, nor flagella, both of which would have complicated the purification of conjugative pili.

Pili were isolated as described. Approximately 75 - 150 g of cells (wet mass) were recovered from the incubation trays in each isolation procedure. From this, less than 100 μg of purified pili (as determined by amino acid analysis) were obtained. The procedure ultimately used in the isolation of the pili proved to be the best compromise between yield and purity of the final product. A final step that involved banding the pili on cesium chloride

density gradients provided pili of higher purity but drastically reduced the overall yield. Treatment of a crude pili preparation with 1 - 2% PEG in the presence of 0.5M NaCl failed to cause the pili to precipitate. Treatment with 10% ammonium sulfate produced unreliable precipitation of the pili from solution, whereas treatment with 20% ammonium sulfate resulted in excessive contamination of the pili with other components of the crude extract.

Pili present in a crude preparation that was subjected to cesium chloride density gradient centrifugation produced a band in the gradient with a refractive index of 1.3630-1.3644 (at 20°C), corresponding to a density of 1.31-1.32 g/ml. Several different pili samples were examined, and all had refractive indices within this range. This value is somewhat higher than that for F pili, which has been reported in the range of 1.20 - 1.26 g/ml (11). However, this density is comparable to that observed for RP4 pili (L. S. Frost, personal communication).

Concentration of pili in the purified samples was determined by amino acid analysis. Other techniques proved to be unsuitable. A mini-method based on the assay of Lowry *et al.* (78) produced no measurable color change compared to negative control samples, while the BioRad protein assay kit proved to be unsuitable due to the minute quantities of protein involved.

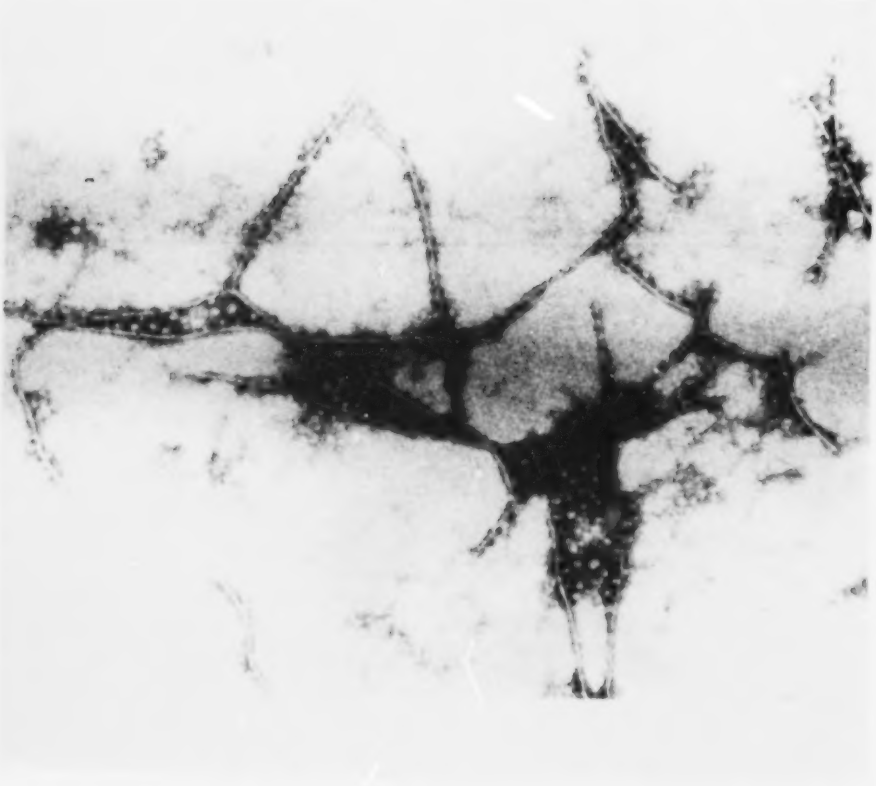
Purified pili was examined at various stages of the purification process by electron microscopy. Each successive purification step reduced the

relative amount of contaminating material present in the sample, but also reduced the quantity of pili as well. A small amount of contaminating material appeared to remain associated with the pili regardless of the number of rounds of ammonium sulfate precipitation attempted. A sample of purified H pili, as observed by electron microscope, is shown in Figure VI-1.

Conjugative pili expressed by both pDT1942 (the derepressed derivative of R27) and pKFW99 (a derepressed derivative of the IncHI2 plasmid R477-1, 166) were isolated for further analysis.

Figure VI-1 Electron micrograph of purified H pili

Purified pili sample was applied to formvar coated grids and stained with 1% sodium phosphotungstate. 65,000X.



B. SDS-PAGE analysis of purified conjugative pili

Purified pili samples were examined by SDS-PAGE analysis, as described. Initially, standard gels (14% acrylamide), cast according to the method of Laemmli (71) were used. Under these conditions, no distinct protein bands were visible in the purified product. Staining with either Coomassie Blue or silver failed to produce more than a faint smear at the bottom of the gel. Increasing the acrylamide concentration to 20% failed to resolve the problem.

It was suspected that the pilin protein could be very small and therefore not well resolved with standard SDS-PAGE procedures. Therefore, a high resolution acrylamide gel system was used, as described. Under these conditions, a well-resolved protein band was visible in the gels with an apparent molecular weight of approximately 3.0-kDa (see Figure VI-2). This band was easily visible when stained with silver. Staining with Coomassie Blue produced extremely faint staining that was difficult to discern.

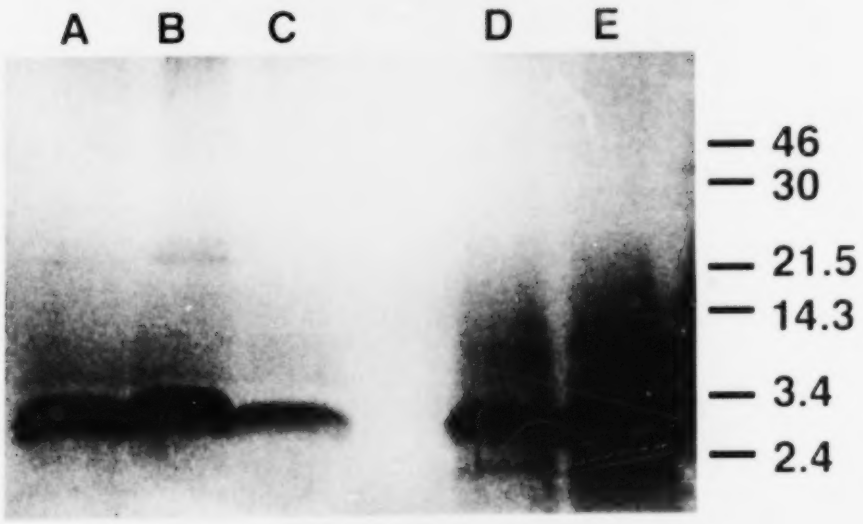
Occasionally, some preparations of pili produced a double band on SDS-PAGE. The two pilin bands had apparent molecular weights of 3.0-kDa and 3.2-kDa respectively. No other bands were apparent in the cleanest pili preparations.

Conjugative pili isolated from *E. coli* harboring a derepressed derivative of R477-1 were also examined by high resolution SDS-PAGE and compared to the R27 pilin. The R477-1 pilin protein appeared on SDS

acrylamide gels as a small protein of approximately 3.2-kDa (slightly larger than the R27 pilin), with staining properties similar to those of R27 pilin (i.e. very poor Coomassie Blue staining).

Figure VI-2 SDS-PAGE analysis of purified H pili

Purified pili were subjected to electrophoresis on high resolution SDS polyacrylamide gels as described. Gels were silver stained and overdeveloped to assist in the detection of contaminants. Lanes A, B, C - purified pili from R477-1 (IncHI2); lanes D and E - purified pili from R27 (IncHI1). Molecular weight markers are indicated in kDa.


A
B
C
D
E
46
30
21.5
14.3
3.4
2.4

C. Amino acid analysis of conjugative pili

Samples of the purified R27 and R477-1 pili were subjected to amino acid analysis, as described. Since none of the methods used to determine protein concentration of the purified pili provided meaningful results, protein concentration was estimated from band intensity of samples subjected to SDS-PAGE. A sample containing an estimated 500 - 600 picomoles of pili, suspended in Milli-Q water, was analyzed. The results of this analysis are shown in Table VI-1.

Table VI-1 Amino acid analysis of pilin protein

Amino Acid[a]	Number of Residues[b]	
	R27 pilin (IncHI1)	R477-1 pilin (IncHI2)
Asx	3	4
Thr	2	2
Ser	4-5	1-2
Glx	2	3
Gly	4	4
Ala	2	2
Val	2	3-4
Ile	1	1
Leu	2	2
Lys	2	2
Total # of residues	24-25	24-26
Calculated molecular weight	2.7-2.9 kDa	2.8-3.0 kDa

[a]Amino acid abbreviations are listed in Appendix 2. Unlisted amino acid residues did not appear in detectable quantity in the amino acid analysis.

[b]Number of residues present are listed at the nearest whole molar ratio.

D. Amino acid sequence analysis of conjugative pili

Numerous attempts at determining a portion of the amino acid sequence of the pilin protein were made. Initially, a sample of the purified pili (500 pmol, suspended in Milli-Q water) was submitted for N-terminal sequence analysis. No PTC-amino acids could be detected in the first five cycles of the procedure. Both the R27 pili and R477-1 pili behaved similarly when subjected to N-terminal sequence analysis.

Additional samples of pili or pilin protein were submitted for sequencing, including a sample dissolved in 50% trifluoroethanol (v/v), and samples of pilin protein blotted to PVDF membrane after electrophoresis through SDS acrylamide gels. None of these samples yielded any N-terminal sequence data, including pilin samples containing both pilin bands observable on SDS-PAGE.

In addition to this, several samples of purified pili (30 - 50 μg) were subjected to mass spectroscopic analysis by ion spray mass spectroscopy. Samples were submitted in Milli-Q water, 10% acetic acid, or 50% trifluoroethanol. None of the pilin samples produced a signal in the mass spectrometer.

E. Pilin protein proteolytic digestion

Based on the amino acid analysis of purified R27 pili, attempts were made to proteolytically cleave the pilin monomer to expose free N-termini for amino acid sequencing, and to construct a peptide map of the protein. Since the protein was found to contain no methionine residues, the cleavage agent of choice, cyanogen bromide, could not be used. Hence, the enzymes trypsin (cleaves at the carboxyl side of arginine and lysine residues) and endoproteinase Glu-C (cleaves at the carboxyl side of glutamic acid residues) were selected for proteolytic digestion of the pilin monomer. The amino acid analysis suggested the presence of two cleavage sites for trypsin, and up to two cleavage sites for endoproteinase Glu-C.

Both urea (8 M) and guanidine-HCl (6 M) were used separately as denaturing agents prior to digestion with the selected enzymes. The digestions were performed as described, for time periods ranging from 1 hour to 24 hours. The ratio of enzyme to substrate was varied from 1:200 to 1:10. In no case could any digestion products be discerned on SDS acrylamide gels. Separation of digestion products by reverse-phase HPLC also showed the presence of only one detectable peptide. Samples of digested protein were subjected to amino acid sequencing procedures, which produced no amino acid sequence.

F. Western blot analysis of pilin protein

Purified R27 pili were used as an antigen to elicit antisera in a New Zealand White rabbit. 20 - 30 μg samples of pili were prepared and injected on a periodic basis as described. Antibody titre was determined by ELISA at each booster. Serum was collected by cardiac puncture when the titre appeared to have leveled off. Final serum titre was approximately 1:10000.

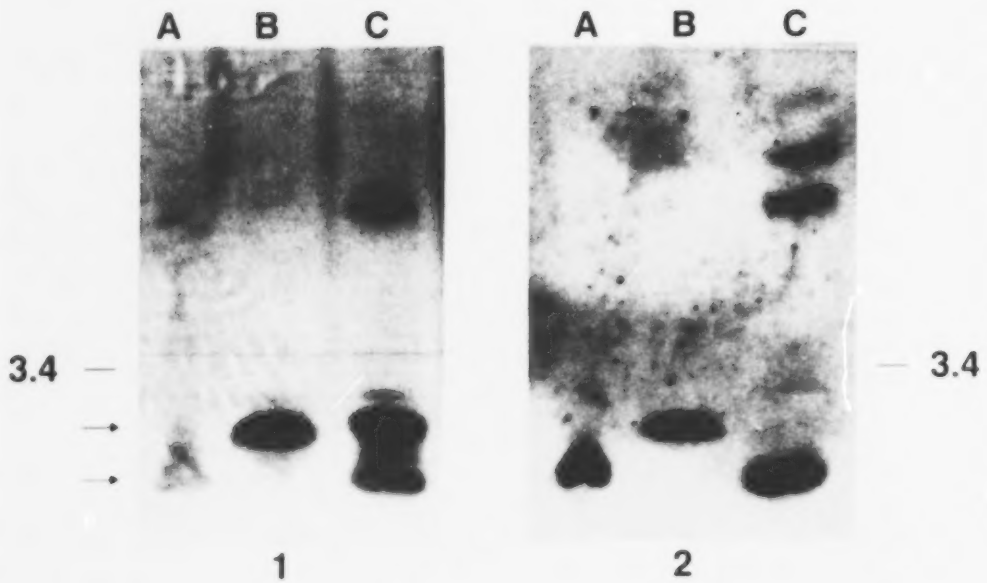
Whole cell protein extracts were prepared from *E. coli* strains JE2571-1 or RG192 harboring plasmid pDT1942 (the derepressed derivative of R27), grown at 28°C. Whole cell protein extracts and purified pili samples were then subjected to SDS-PAGE and electroblotted to nitrocellulose membranes as described. Anti-pili antisera was diluted 1:1000 (for colorimetric detection) or 1:5000 (for enhanced chemiluminescent detection) and used as a probe for the detection of pilin proteins bound to the membrane.

Initial results using unabsorbed antisera and whole cell protein extracts demonstrated that the antisera contained antibodies that recognized and bound to a wide array of *E. coli* bacterial proteins, in addition to the pilin protein. In fact, the cross-reactivity was extensive enough to partially obscure the detection of pilin monomer from a whole cell extract. Because of this, the antisera was absorbed with an acetone powder of the *E. coli* strain used in the original purification of the R27 pili (JE2571) in an attempt to remove some of the cross-reacting antibodies. This treatment reduced, but did not eliminate, the cross-reactivity in the antisera.

Figure VI-3 shows a photograph of an immunoblot depicting hybridization of the anti-pili antisera to purified pili. As can be seen, the antisera reacts with both HI1 and HI2 pilin protein. In addition, only the lower band in samples of HI1 pilin with a doublet pilin band reacts with the antisera.

Figure VI-3 Immunoblot hybridization of anti-pili antisera

Purified pili samples were subjected to electrophoresis on high resolution SDS acrylamide gels, electro-blotted to nitrocellulose, and probed with anti-pili antisera. Hybridization was detected by enhanced chemiluminescence. Panel 1 - silver stained gel; panel 2 - immunoblot. Lane A - purified R27 pili (IncHI1); lane B - purified R477-1 pili (IncHI2); lane C - partially purified R27 pili (IncHI1). The position of the 3.4-kDa molecular weight standard is marked, as well as arrows indicating the positions of the H pilin bands.


A
B
C
3.4
A
B
C
3.4
1
2

G. Western blot analysis of pilin protein produced by other H plasmids

In order to compare the production and size of pilin protein from various other IncH plasmids, whole cell protein extracts were prepared from numerous *E. coli* strains harboring a variety of H plasmids (all grown at 28°C). Approximately 5-10 μg of protein from each sample was subjected to electrophoresis on SDS polyacrylamide gels and compared to protein samples from a strain of *E. coli* carrying pDT1942. The results of this analysis are summarized in Table VI-2 and examples are shown in Figure VI-4.

Most of the strains tested in this manner failed to produce detectable amounts of pilin protein of a size comparable to the R27 pilin. The exceptions included derepressed derivatives of two IncHI2 plasmids, R478 and R477-1. Neither of the strains carrying an IncHII plasmid produced detectable levels of an antigenically related pilin protein.

Figure VI-4 H pilin synthesis from various IncH plasmids.

Whole-cell proteins were prepared and subjected to electrophoresis on high resolution SDS acrylamide gels. After electro-blotting, membranes were probed with anti-pili antisera and detected by colorimetric means. Lane A - pKFW100 (IncHI2), Lane B - R478 (IncHI2), Lane C - MIP233 (IncHI3), Lane D - pHH1508a (IncHII), Lane E - pHH1457 (IncHII), Lane F - pDT1942 (R27drd), Lane G - no plasmid. Molecular weight standards are indicated in kDa, and pilin band positions are indicated by arrows.

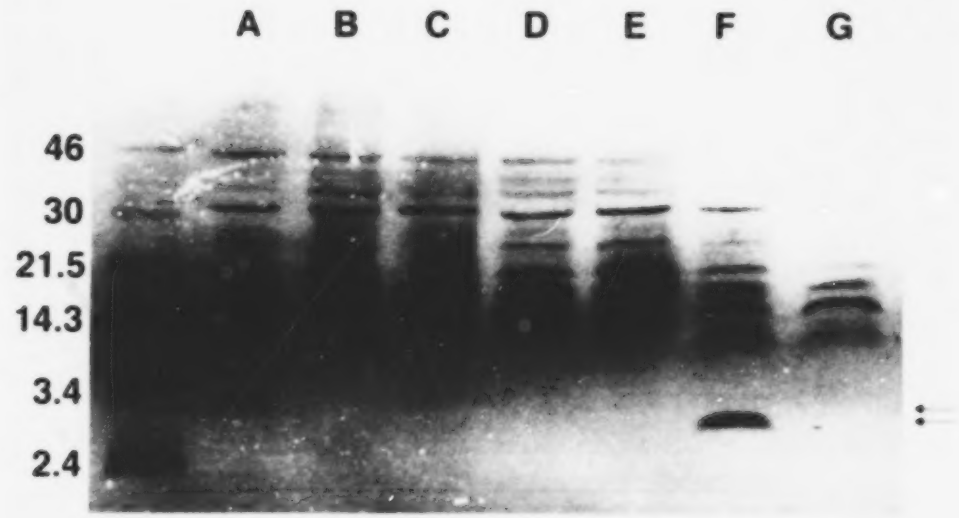

A
B
C
D
E
F
G
46
30
21.5
14.3
3.4
2.4

Table VI-2 Pilin proteins produced by IncH plasmids

Plasmid	Inc Group	Pilin Protein[a]	
		Relative Band Intensity	Apparent Molecular Weight
R27	HI1	-	-
pDT1942 (R27drd)	HI1	++	3.0-kDa
R478	HI2	-	-
pKFW100 (R478drd)	HI2	+	3.2-kDa
R477-1	HI2	-	-
pKFW99 (R477-1drd)	HI2	++	3.2-kDa
MIP233	HI3	-	-
pHH1508a	HII	-	-
pHH1457	HII	-	-

[a]Pilin protein production was ascertained from immunoblots of whole cell protein probed with anti-H pili antisera. Relative band intensities reflect the presence and strength of reaction with a low molecular weight protein not present in plasmid-free cells. The apparent size of the pilin protein as judged from molecular weight size standards is listed.

H. Temperature effects on pilin synthesis

Since plasmids of the IncHI group transfer at very low or undetectable frequency at temperatures above 35°C, the effect of temperature on the synthesis of pilin protein was investigated. Electron microscopic analysis of *E. coli* strain JE2571-1 carrying plasmid pDT1942 revealed the presence of 1-2 H pili on most cells in a logarithmic-phase culture incubated at 28°C. When grown at 37°C, logarithmic-phase cells possessed no pili, as evidenced by electron microscopic analysis.

Western blot analysis of whole cell protein extracts from strain RG192(pDT1942) showed that pilin protein is not present in detectable quantity in cells grown at 37°C. To investigate this phenomenon further, temperature-shift experiments were carried out. An overnight culture (BHI broth) of strain RG192(pDT1942) was diluted 1:100 in fresh BHI broth and incubated at either 28°C or 37°C until cell density reached an $O.D._{600}$ of 0.20 - 0.25. A sample of the culture was removed and stored on ice and the remainder of the culture was shifted to the lower or higher temperature (i.e. 28°C $\Rightarrow$ 37°C, or 37°C $\Rightarrow$ 28°C). Samples were then taken periodically from the culture over the next several hours. Each sample was immediately placed on ice, and prepared for SDS-PAGE analysis. All samples were equalized for cell density (as measured by optical density at 600 nm) and subjected to SDS-PAGE and Western blot in order to determine comparative pilin concentration. The results are depicted in Figure VI-5 and Figure VI-6.

Pilin monomer levels gradually diminish over the course of the experiment when temperature is shifted from 28°C to 37°C. With a temperature shift in the opposite direction, pilin monomer first appears at detectable levels approximately 90 minutes after the temperature shift, and maximal synthesis occurs 120 minutes after the shift.

Figure VI-5 Effect of a temperature shift (37°C to 28°C) on steady-state pilin levels

Protein samples were prepared as described and subjected to electrophoresis on high resolution SDS polyacrylamide gels. After electroblotting to nitrocellulose, the membrane was probed with anti-H pili antisera. Bound antisera was detected by enhanced chemiluminescence. Elapsed time after shift for each sample is indicated below. Molecular weight standards are indicated in the figure.

Lane A - 0 min.

Lane B - 1 min.

Lane C - 5 min.

Lane D - 15 min.

Lane E - 30 min.

Lane F - 60 min.

Lane G - 90 min.

Lane H - 120 min.

Lane I - 150 min.

Lane J - 180 min.

Lane K - 240 min.

Lane L - purified R27 pili

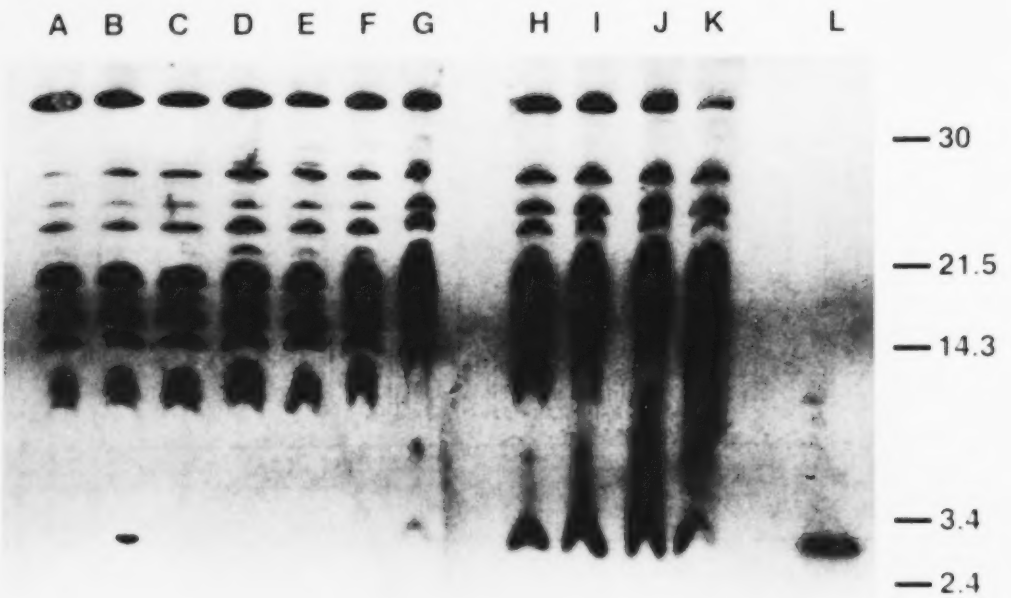

A B C D E F G H I J K L
30
21.5
14.3
3.4
2.4

Figure VI-6 Effect of a temperature shift (28°C to 37°C) on steady-state pilin levels

Protein samples were prepared as described and subjected to electrophoresis on high resolution SDS polyacrylamide gels. After electroblotting to nitrocellulose, the membrane was probed with anti-H pili antisera. Bound antisera was detected by enhanced chemiluminescence. Elapsed time after shift for each sample is indicated below. Molecular weight standards are indicated in the figure.

Lane A - 0 min.

Lane B - 30 min.

Lane C - 60 min.

Lane D - 90 min.

Lane E - 120 min.

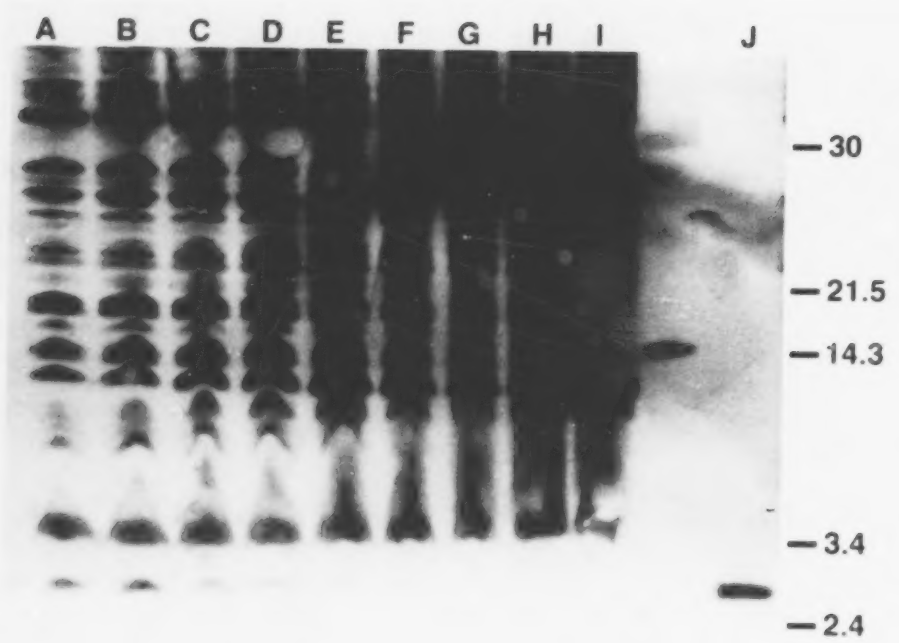
Lane F - 150 min.

Lane G - 180 min.

Lane H - 240 min.

Lane I - 300 min.

Lane J - purified R27 pili


A
B
C
D
E
F
G
H
I
J
30
21.5
14.3
3.4
2.4

I. Pulse-chase labelling of pilin protein

Since the procedures detailed in the previous section revealed steady-state pilin levels only, a series of pulse-chase experiments were devised and carried out in an attempt to ascertain the effect of temperature shifts on the *de novo* synthesis of pilin monomer. Based on the amino acid analysis of purified pili, the pilin protein possessed no sulfur-containing amino acids (Cys, or Met), so a ^{35}S-labelled amino acid could not be used in the experiment. As a result, ^{3}H-leucine was employed as a label in the pulse phase of the procedure.

Initial results for the procedure were not encouraging. It appeared that insufficient label was incorporated to allow the pilin protein to be seen on autoradiographs of the polyacrylamide gels used to resolve proteins labelled in the pulse phase of the experiment. Numerous changes were made to the procedure in an attempt to enhance labelling of the pilin. These included the use of a richer media for growth (both LB and Mueller Hinton broth), increasing the pulse time (up to 5 min.), and increasing the amount of label added to the growth media. None of the measures taken resulted in more than very minimal incorporation of label into pilin monomer.

J. Pilin expression from isolated fragments of R27

E. coli strains carrying large fragments of R27 cloned into cosmid and plasmid vectors were tested for pilin production by isolating total cellular protein from logarithmic phase cultures grown in BHI broth at 28°C. Pilin protein was assayed by Western immunoblot, using polyclonal anti-pili antisera, as described. Descriptions of the fragments tested can be found in Figure I-2. None of the cloned fragments tested in this manner exhibited any detectable pilin synthesis.

Chapter VII Mutagenesis and nucleotide sequence of Tra genes encoded by R27

A. Mini-Tn10 insertion mutagenesis

Plasmid pNK2884 was used as a delivery vehicle for transposon insertion mutagenesis of pDT1942, as described in Materials and Methods. *E. coli* strain J53-1 (Nal^R) was selected as the host strain for transposition, based on initial testing that demonstrated the highest rates of transposition to occur in this strain. Strain RG192 (Rif^R) was used as a recipient in the experiment. Approximately 3,500 Cm^R transconjugants were screened for transfer proficiency. Of these, 45 proved to be transfer deficient in overnight matings, suggesting a rate of 1.3 per 100 insertions resulted in a Tra^- phenotype. Numerous others exhibited reduced, but detectable transfer in overnight matings.

B. Mapping of Tra^- insertion mutants

Each Tra^- mutant of pDT1942 generated by the insertion of mini-Tn10 was examined to determine the location of the mini-transposon within the plasmid. In each case, plasmid DNA was isolated and digested with the restriction endonucleases *Sal*I, *Xba*I, and *Apa*I. Restricted plasmid DNA was subjected to electrophoresis in agarose gels (0.5%, TAE), along with restricted

plasmid DNA from pDT1942 for comparison. Since the enzymes chosen for this analysis do not cut within the mini-Tn10 transposon, transposon insertion was evidenced as an increase in size of one of the restriction digest fragments.

In addition, digested plasmid DNA was blotted to nitrocellulose and hybridized with a radiolabelled DNA fragment encompassing the mini-Tn10 region of the original transposon delivery vector (pNK2884). Hybridization patterns, taken with changes in restriction digest patterns, provided unequivocal assignment of the location of insertion. Figure VII-1 shows a typical example of this analysis.

Insertion locations in the R27 plasmid that produced a Tra$^-$ phenotype are indicated in Figure VII-2. As can be seen, the insertions are scattered through a large proportion of the plasmid, but lie broadly within the two previously defined Tra regions, Tra1 and Tra2.

Figure VII-1 Location of mini-Tn10 insertions

Plasmid DNA was digested with restriction endonucleases and subjected to electrophoresis through agarose gels (0.5%, TAE buffer). DNA was blotted to nitrocellulose and probed with a ^{32}P-labelled mini-Tn10 probe (pNK2884). After hybridization, blots were exposed to X-ray film overnight. Ethidium bromide stained gel is shown to the left, and autoradiograph to the right. Molecular size standards are indicated in kb. Arrows indicate fragments with transposon insertions.

Lane A - pNK2884

Lane B - insertion mutant 9, S*al*I-cut

Lane C - insertion mutant 20, S*al*I-cut

Lane D - insertion mutant 3, S*al*I-cut

Lane E - pDT1942, S*al*I-cut

Lane F - insertion mutant 9, A*pa*I-cut

Lane G - insertion mutant 20, A*pa*I-cut

Lane H - insertion mutant 3, A*pa*I-cut

Lane I - pDT1942, A*pa*I-cut

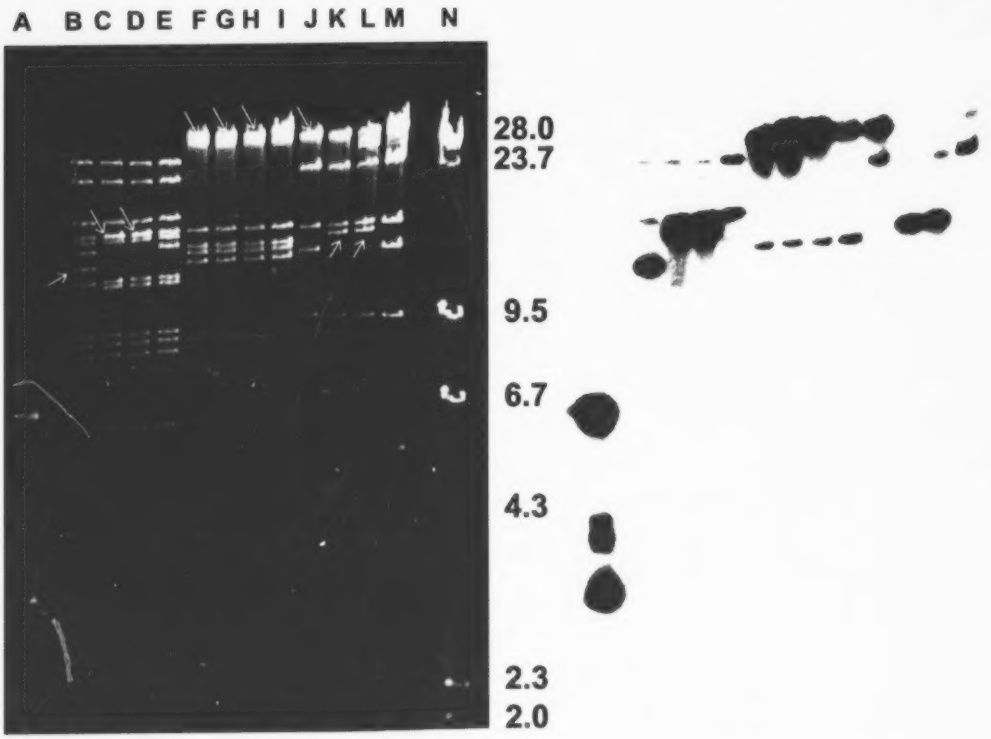
Lane J - insertion mutant 9, X*ba*I-cut

Lane K - insertion mutant 20, X*ba*I-cut

Lane L - insertion mutant 3, X*ba*I-cut

Lane M - pDT1942, X*ba*I-cut

Lane N - λ, H*ind*III-cut


A B C D E F G H I J K L M N
28.0
23.7
9.5
6.7
4.3
2.3
2.0

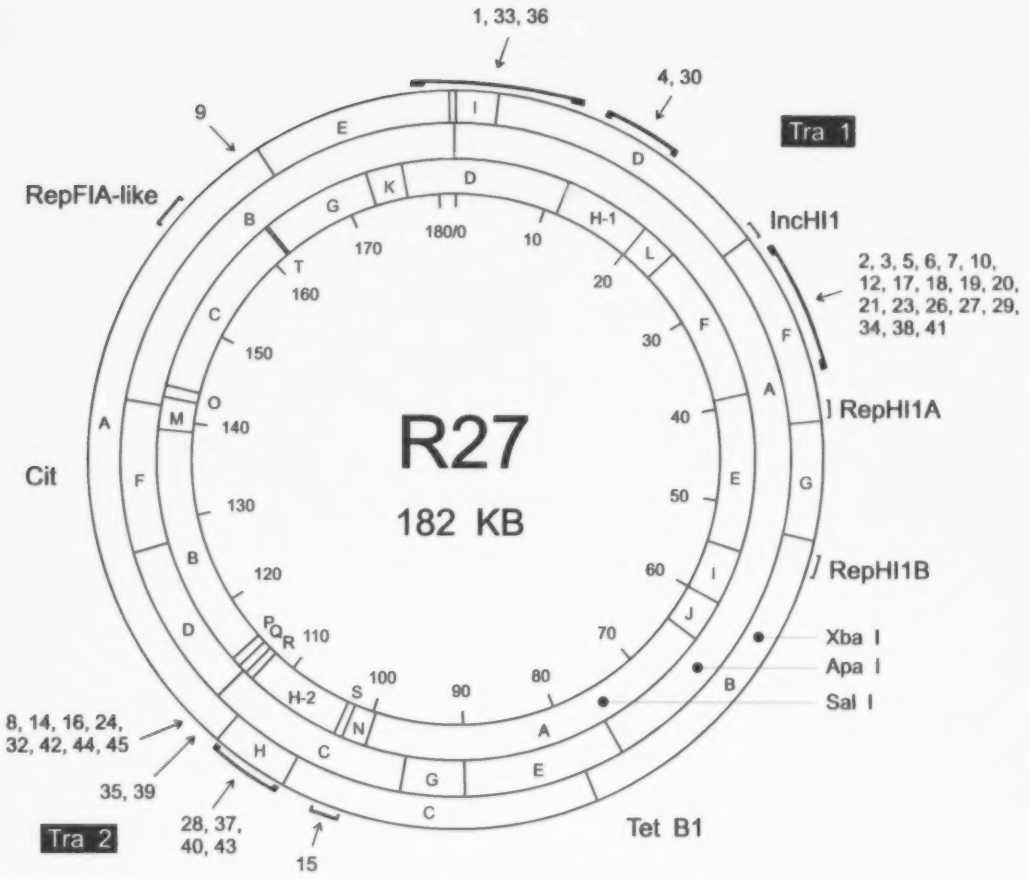


Figure VII-2 Mini-Tn10 insertion sites

The physical and genetic map of R27 is depicted. For details on designations, refer to Figure I-1 and Figure IV-2. Locations of mini-Tn10 insertions that produce a Tra⁻ phenotype are indicated by numbers around the outside circle.

C. Pilin expression of Tra⁻ mutants

Each of the mini-Tn10 insertion mutants was assayed for the production of pilin protein by subjecting samples of whole cell proteins isolated from logarithmic phase cells to SDS-PAGE. A single isolated colony of each mutant was inoculated to BHI broth and incubated overnight with aeration at 28°C. The culture was diluted 1:100 in fresh media and incubation continued for an additional four hours. A one ml sample was taken and total cellular proteins solubilized as described. 10 µl of this was subjected to electrophoresis through high resolution SDS acrylamide gels. The gels were then electroblotted to nitrocellulose, and probed with absorbed rabbit anti-pili antisera. Bound antisera was detected by colorimetric methods, using alkaline phosphatase-conjugated goat anti-rabbit IgG as the secondary antibody.

A portion of these results are shown in Figure VII-3, and completely summarized in Table VII-1. Among the 40 mutants analyzed, only four showed an altered pilin protein level: three produced detectable, but greatly reduced levels of pilin protein, while one failed to produce any detectable pilin protein at all.

Figure VII-3 Pilin synthesis in R27 Tra⁻ mutants

Protein samples were subjected to electrophoresis on high resolution gels and electroblotted to nitrocellulose. Membranes were probed with anti-H pili antisera and reactive proteins were detected by colorimetric methods. Molecular weight standards are indicated in kDa. The arrow indicates the pilin protein. Samples loaded are as follows (R27 Tra⁻ mutant insertion sites are indicated in Figure VII-2).

Lane	R27 Tra⁻ Mutant	Lane	R27 Tra⁻ Mutant
A	45	K	39
B	20	L	38
C	44	M	34
D	43	N	12
E	9	O	33
F	2	P	37
G	32	Q	29
H	42	R	36
I	41	S	35
J	40		

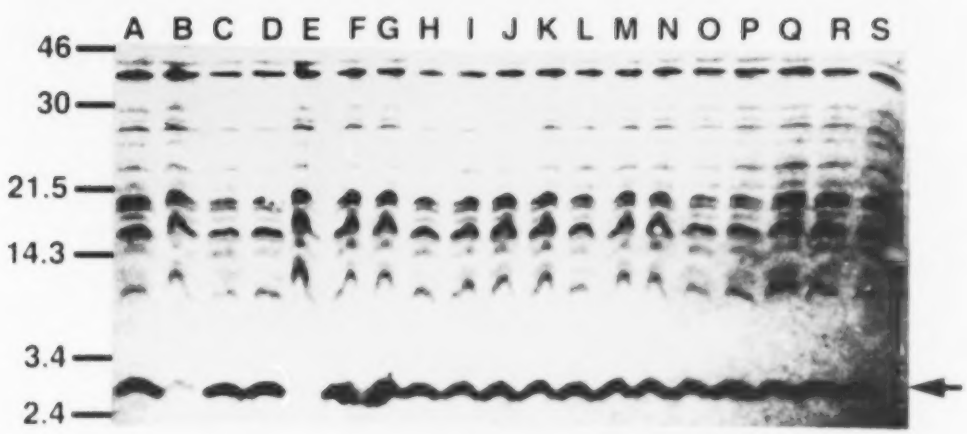

A B C D E F G H I J K L M N O P Q R S
46
30
21.5
14.3
3.4
2.4

Table VII-1 Pilin production by R27 Tra⁻ mutants

Tra⁻ Mutant[a]	*Sal*I Fragment[b]	Pilin[c]	Tra⁻ Mutant	*Sal*I Fragment	Pilin
1	D	++	24	R	++
2	F	++	26	F	++
3	F	+/-	27	F	++
4	H-1	++	28	H-2	++
5	F	++	29	F	++
6	F	++	30	H-1	++
7	F	++	32	R	++
8	R	++	33	D	++
9	G	-	34	F	++
10	F	++	35	Q	++
12	F	++	36	D	++
14	R	++	37	H-2	++
15	N	++	38	F	++
16	R	++	39	Q	++
17	F	++	40	H-2	++
18	F	++	41	F	++
19	F	++	42	R	++
20	F	+/-	43	H-2	++
21	F	+/-	44	R	++
23	F	++	45	R	++

[a,b]Tra⁻ mutant descriptions and locations are as indicated in Figure VII-2.

[c]Pilin expression scored as follows: ++, comparable to pDT1942; +/-, pilin detectable at low level compared to pDT1942, -, no pilin detected.

D. Phage sensitivity of Tra⁻ mutants

In order to ascertain whether any of the mini-Tn10 insertions affecting transfer also affected conjugative pilus production, each Tra⁻ mutant was tested for pilHα plaquing, as described. This phage has been shown to attach to the shaft of the H pilus, and it will produce plaques on *E. coli* harboring transfer de-repressed derivatives of IncH plasmids. Plaque spot tests were performed on three separate occasions, using two different phage stocks. The results of this experiment are summarized in Table VII-2. In each case, similar results were obtained in at least two of three tests.

Spot tests showed reduced or undetectable plaque formation for the majority of mini-Tn10 insertion mutants. Insertions in both Tra1 and Tra2 produced altered phage sensitivity. None of the insertions in either the S*al*I-H1, S*al*I-H2, or S*al*I-N fragments resulted in reduced sensitivity to the H-pilus specific phage. All of the insertions in S*al*I fragments G, Q, and R eliminated plaque formation in spot tests. Variable sensitivity was observed with insertions in the S*al*I fragments D and F. All four insertion mutants with altered pilin expression (3, 9, 20, 21) showed no phage sensitivity in this assay.

Table VII-2 PilHα plaque formation on R27 Tra⁻ mutants

Tra⁻ Mutant[a]	Plaque Formation[b]	Tra⁻ Mutant	Plaque Formation
1	+/-	24	-
2	-	26	-
3	-	27	+/-
4	+	28	+
5	-	29	-
6	-	30	+
7	-	32	-
8	-	33	-
9	-	34	-
10	-	35	-
12	-	36	+/-
14	-	37	+
15	+	38	-
16	-	39	-
17	+/-	40	+
18	-	41	-
19	-	42	-
20	-	43	+
21	-	44	-
23	-	45	-

[a]Insertion locations for Tra⁻ mutants are depicted in Figure VII-2.

[b]Plaque formation was scored as follows: +, clear plaques; +/-, turbid plaques; -, no plaque formation.

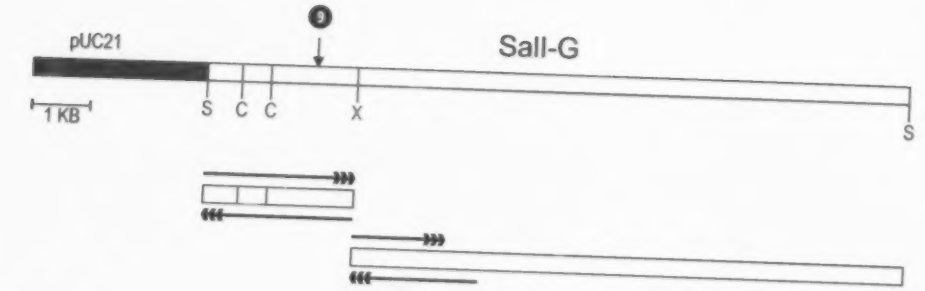
E. Nucleotide sequence of Tra regions of R27

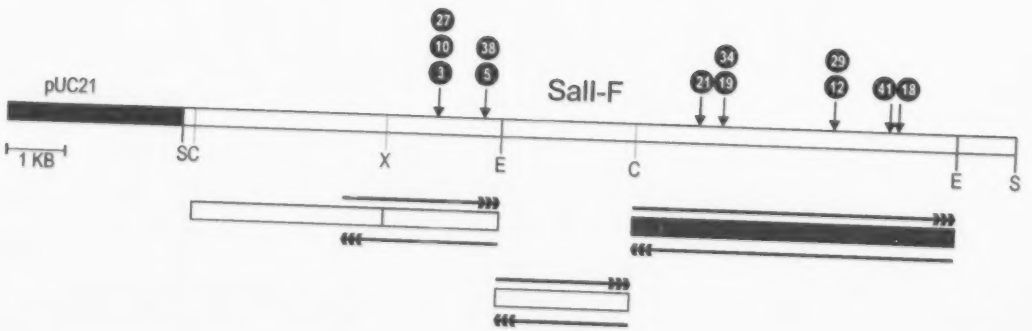
Since the data suggested that the Tra1 region is involved in pilus synthesis, nucleotide sequence analysis of this region was undertaken. Specifically, sections of the *Sal*I-F and *Sal*I-G fragments were selected for sequencing, based on the observation that some insertions in these regions affected pilin synthesis.

Initially, the *Sal*I-F and *Sal*I-G fragments were subcloned to pUC21 from pDT1233 and pDT1602, respectively. Further subcloning was initiated at this point to reduce the size of these fragments to something more manageable for sequencing. Once sufficient subclones were generated, exonuclease III was used to construct nested deletions of each subclone. Deletions were constructed from both ends of each fragment. The deletions allowed the use of relatively few primers in the determination of the nucleotide sequence of the cloned DNA. The construction of subclones and deletions is summarized in Figure VII-4.

Figure VII-4 Subcloning of R27 S*al*I-F and S*al*I-G fragments

Regions of the S*al*I-F and S*al*I-G fragments of R27 subcloned to plasmid vectors (pUC21 and pUCBM20) are indicated below each map. Major restriction sites are indicated (S - S*al*I, C - C*la*I, X - X*ba*I, E - E*co*RI). The arrows above and below each subcloned region indicate the direction and extent of exonuclease III digestion, used in the creation of deletion derivatives for sequencing. The circles above each map indicate insertion sites for Tra$^-$ mutants created with mini-Tn10. Numbers correspond to those in Figure VII-2 and Table VII-1. The 5.5-kb E*co*RI-C*la*I fragment depicted in gray below the S*al*I-F map has been completely sequenced. Nucleotide sequence for other regions is incomplete.


9
pUC21
SalI-G
1 KB
S
C
C
X
S


27
10
38
3
5
34
21
19
29
12
41
18
pUC21
SalI-F
1 KB
SC
X
E
C
E
S

Initially, nucleotide sequence was determined by standard dideoxy-terminated sequence procedures, using double-stranded template (denatured with NaOH), and modified T7 DNA polymerase. Although satisfactory results were obtained using this method, frequent "fall-offs" or strong stops were routinely observed in the sequence. In addition, it was often difficult to read more than 250 - 300 nucleotides of sequence per reaction. Subsequently, nucleotide sequence was determined by cycle sequencing with Taq polymerase. This procedure proved to be vastly superior to Sequenase sequencing. Very few strong stops were observed in the sequence, and typical reaction runs could be read to 450 nucleotides of sequence or more.

Nucleotide sequence deduced from these procedures is presented in Figure VII-5. This sequence covers a 5.5-kb *Eco*RI-*Cla*I fragment subcloned from the *Sal*I-F region of R27, as indicated in Figure VII-4. The sequence presented here has been derived from both DNA strands. Sequence data derived from other regions in the *Sal*I-F and *Sal*I-G fragments has been determined for one strand only, with some sections sequenced from both DNA strands, and for this reason, is not shown in this thesis.

Figure VII-5 Nucleotide sequence of a 5.5-kb region of the S*al*I-F fragment of R27.

The sequence presented corresponds to a 5.5-kb E*co*RI-C*la*I fragment derived from the S*al*I-F region of R27. C*la*I and E*co*RI sites are indicated as overlined bases in the sequence. Potential translation products are indicated below the sequence as ORF1 through ORF8. Sequences with similarity to the consensus Shine-Dalgarno ribosome binding sequence (128) are underlined.

```
 EcoRI  10                  30                  50
GAATTCAAATCAACACGGAGTAATTATGGAACTGACATTGAATACTAACGTTGAAAGAGT
.AsnSerAsnGlnHisGlyValIleMetGluLeuThrLeuAsnThrAsnValGluArgVa
 ORF1
        70                  90                  110
AAAAACTAACGCTTTTGCATTTTTGAAGTCCAAACATGCAAAAGTGCCTTTGTTTATTCT
lLysThrAsnAlaPheAlaPheLeuLysSerLysHisAlaLysValProLeuPheIleLe

       130                 150                 170
CTTGGTTTTAGCAAATTGCAGTTTTGCGTACGCGGGTTCCGATGATGGACGCTTCGGCGA
uLeuValLeuAlaAsnCysSerPheAlaTyrAlaGlySerAspAspGlyArgPheGlyAs

       190                 210                 230
TATCTGGGCCTACATGAGCGAAGCCTTGACTGGTGCGCCGGGTAAAATCATCGCGTGCGG
pIleTrpAlaTyrMetSerGluAlaLeuThrGlyAlaProGlyLysIleIleAlaCysGl

       250                 270                 290
CATGTTGTTCTCgGTGGCTTATTTCGGCGTTGTAAAACCTAACCTTGGTTTGGCGCTGGT
yMetLeuPheSerValAlaTyrPheGlyValValLysProAsnLeuGlyLeuAlaLeuVa

       310                 330                 350
ATCAGCATTAATGATGCTGGTTATGGCAAACGGTGAAAAAATTATCAGCTCGTTCCTGGA
lSerAlaLeuMetMetLeuValMetAlaAsnGlyGluLysIleIleSerSerPheLeuAs

       370                 390                 410
TGCTGGTATTCCTCTGTGATTTGTTAGAACAATAACAATGAGGGGGATATTCCCCCTCTC
pAlaGlyIleProLeu                        MetArgGlyIlePheProLeuSe
                                        ORF2
       430                 450                 470
TGGAGTTTTTATGACAAGCCAGTACGAGATACCGCCACATACATATCGCTTTCCATACAG
rGlyValPheMetThrSerGlnTyrGluIleProProHisThrTyrArgPheProTyrAr

       490                 510                 530
AATAAATATGCCGTTACTAATTCTATTCTGGGATGCAAAGCAATTGGGTATAACATTCGT
gIleAsnMetProLeuLeuIleLeuPheTrpAspAlaLysGlnLeuGlyIleThrPheVa

       550                 570                 590
GACTATCGCGAGCGGTAATATTTTTGATTTCTTTATAACTTCGGTAGTTGTGGCGGTGGT
lThrIleAlaSerGlyAsnIlePheAspPhePheIleThrSerValValValAlaValVa

       610                 630                 650
GTTCTGGTTTGCATATAAGAAAGCAGCAGAGGAAGGGATTAGAGGAAAACTAAAACACAA
lPheTrpPheAlaTyrLysLysAlaAlaGluGluGlyIleArgGlyLysLeuLysHisLy

       670                 690                 710
ACTGTGGTGGTACGGCTTTTTTCCTGGAAAATCGGTGTTTAGTAGTCGTTACTTTACCGA
sLeuTrpTrpTyrGlyPhePheProGlyLysSerValPheSerSerArgTyrPheThrAs
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       730                   750                   770
TCCATTTATCAGAAACCTCTATTCTTGATGGAGCTAAGAATGAAACTTCTCAGCAGGTTA
pProPheIleArgAsnLeuTyrSer                MetLysLeuLeuSerArgLeu
                                         ORF3
       790                   810                   830
AAAATTCCATTCATTAAAAATTCCAGTAATGGCGATTTTGATAACAAGGATGAGACTTCG
LysIleProPheIleLysAsnSerSerAsnGlyAspPheAspAsnLysAspGluThrSer

       850                   870                   890
CAAAAAGGAGGTGAAGAAGTAAAAGGAAGTTTTCTGGATTCAAAAGCACGCTTTAGTAAA
GlnLysGlyGlyGluGluValLysGlySerPheLeuAspSerLysAlaArgPheSerLys

       910                   930                   950
GAAATTGAAGGTTCTGAAATTGGAATTACATATAGTGCATTGATTAAACGCGACGAAAAA
GluIleGluGlySerGluIleGlyIleThrTyrSerAlaLeuIleLysArgAspGluLys

       970                   990                  1010
CTCCTACGTGTGAATACAGTTGCAATGATCATTATTGGGGTTCTGGTTGTAAAAAATCAA
LeuLeuArgValAsnThrValAlaMetIleIleIleGlyValLeuValValLysAsnGln

      1030                  1050                  1070
TTTCTCACTGATCCTGTGACTATAGTGCTACCGCCAAATATGACGGAAGAAGTCAAAGTT
PheLeuThrAspProValThrIleValLeuProProAsnMetThrGluGluValLysVal

      1090                  1110                  1130
GTTGGAAATAAAGCCTCAGAATCATATAAAACGCAGTGGGCCTTATTCTTTAGTACTTTG
ValGlyAsnLysAlaSerGluSerTyrLysThrGlnTrpAlaLeuPhePheSerThrLeu

      1150                  1170                  1190
ATTGGAAACATAAACCCAACAAACATCGGTTTTGTTACAACAACTATATTGGATGCGCTT
IleGlyAsnIleAsnProThrAsnIleGlyPheValThrThrThrIleLeuAspAlaLeu

      1210                  1230                  1250
TCTCCGGATTTACAGGCAAAGACCCGGGAATCACTACAGCAGCAAACCAATATAATGCAG
SerProAspLeuGlnAlaLysThrArgGluSerLeuGlnGlnGlnThrAsnIleMetGln

      1270                  1290                  1310
GCTCGTGGTGTAGAGCAGTCATTTAAGCCGATCGATATGTATTACGACACCAAAAATGAC
AlaArgGlyValGluGlnSerPheLysProIleAspMetTyrTyrAspThrLysAsnAsp

      1330                  1350                  1370
ATGGTCTATGTCTGGGGAACAAAGTCAACTCGTTTGATTAATGTTCCGGACAAAACTGAA
MetValTyrValTrpGlyThrLysSerThrArgLeuIleAsnValProAspLysThrGlu

      1390                  1410                  1430
TCATCCAAATGGACGTTTGAATGGGTCCTGGGAATGAAAAATGGACGACCGCGCATAGCC
SerSerLysTrpThrPheGluTrpValLeuGlyMetLysAsnGlyArgProArgIleAla
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      1450                1470                1490
TATGTGAACCAGTATTCAGGTACACCAAATATTAAGAAAATTACAATCAACGGCAAAGAA
TyrValAsnGlnTyrSerGlyThrProAsnIleLysLysIleThrIleAsnGlyLysGlu

      1510                1530                1550
CAACTGGCAACACTCGATAATCCGCCGCCGTCAATAGGTAACTAATTATGACTTCAAAAA
GlnLeuAlaThrLeuAspAsnProProProSerIleGlyAsn     MetThrSerLysL
                                                ORF4
      1570                1590                1610
AGGTTTTTCACTGCACCGCGATTACAGCAGTGATTTTGCTGGCAACTTCGAATGTGGTAT
ysValPheHisCysThrAlaIleThrAlaValIleLeuLeuAlaThrSerAsnValValP

      1630                1650                1670
TTGCTGAAGACTATCAGTTACCGGCGACAGTAAATAATCCGGTAGTTATGCCTGTCGGGG
heAlaGluAspTyrGlnLeuProAlaThrValAsnAsnProValValMetProValGlyA

      1690                1710                1730
CCGATGAATTTCAGAACGGTGTGAAAAATGCGATAATTAAAGACACTGGCACTACGACTG
laAspGluPheGlnAsnGlyValLysAsnAlaIleIleLysAspThrGlyThrThrThrG

      1750                1770                1790
AGACCGTACAAGCAACAAAACCGACAACTGCTCTGCCAAGTCTTTCACCTGCAAGCAGTG
luThrValGlnAlaThrLysProThrThrAlaLeuProSerLeuSerProAlaSerSerA

      1810                1830                1850
CTTCTCCCGCAGTCAATGAAATAACCGGGGCGTTGTCAAATAACCCTACTCTAGCTGGTT
laSerProAlaValAsnGluIleThrGlyAlaLeuSerAsnAsnProThrLeuAlaGlyT

      1870                1890                1910
ACCAGCAACAGGTTAAATCAGGAAATTTTGATAGTTACGGAAGACCAGCAGGGAAAGAAC
yrGlnGlnGlnValLysSerGlyAsnPheAspSerTyrGlyArgProAlaGlyLysGluP

      1930                1950                1970
CTCAACAAAATGCACAAAGTACCGGGTCCCCTTCAAAGGCAGATGAACTTTATGTTGAAG
roGlnGlnAsnAlaGlnSerThrGlySerProSerLysAlaAspGluLeuTyrValGluA

      1990                2010                2030
CACGTAATCGCTATAAAGAAGTACAACGAGTAAACGTTCCGCCTGGTGGCAATATTGTAC
laArgAsnArgTyrLysGluValGlnArgValAsnValProProGlyGlyAsnIleValL

      2050                2070                2090
TCCCGGTTTCTCGGGGGCTGCAAAACAGAATTTCGACTTCATTTAAAAATGCATCTGTAA
euProValSerArgGlyLeuGlnAsnArgIleSerThrSerPheLysAsnAlaSerValS

      2110                2130                2150
GTACTTCAACGCCAGCTGATGAAGCCAGTATTTTCGTTAATGGCGGGGATGTTTTTATTT
erThrSerThrProAlaAspGluAlaSerIlePheValAsnGlyGlyAspValPheIleS
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      2170                2190                2210
CAACAAATACAGATAAGCCTATAGGAATTATGCTTTCAGAAGATTCAGTTCCTGAATCTA
erThrAsnThrAspLysProIleGlyIleMetLeuSerGluAspSerValProGluSerT

      2230                2250                2270
CTTATAACCTGACACTGGTTCCACTGGATGTACCAGGGGCCATGATTTCAGTAACAACTT
hrTyrAsnLeuThrLeuValProLeuAspValProGlyAlaMetIleSerValThrThrS

      2290                2310                2330
CTTTGAGCCCGACAATGCAGGCAAAACGAGAAACTTCTCTTGATAAACAAAATTATGATG
erLeuSerProThrMetGlnAlaLysArgGluThrSerLeuAspLysGlnAsnTyrAspG

      2350                2370                2390
AGATGCTGGCACGCTCACAATCAGAAGAGTTGACTCCTTCAGACCCTCGGCAGGATGACC
luMetLeuAlaArgSerGlnSerGluGluLeuThrProSerAspProArgGlnAspAspH

      2410                2430                2450
ATAAACAGCGGATCATTGATTTGTTGACTCCGGTTGCACTGGGTGAGGTTCCTTCTGGTT
isLysGlnArgIleIleAspLeuLeuThrProValAlaLeuGlyGluValProSerGlyP

      2470                2490                2510
TCAGTTTACAAGAAGACCGCTTGTCACGCATACCAAGCTCAGAGCAGTCCCCATGTAATT
heSerLeuGlnGluAspArgLeuSerArgIleProSerSerGluGlnSerProCysAsnP

      2530                2550                2570
TCAATATGTACGCCAAGTTAGGTCAAAGACTGGTGGGATCTCGGGAATTGATAGATGTCG
heAsnMetTyrAlaLysLeuGlyGlnArgLeuValGlySerArgGluLeuIleAspValV

      2590                2610                2630
TTCTTGTTAAGAATGATAAGCCTTACGGACAGGTAGTGGCTGATCAGCAATGTATAACTG
alLeuValLysAsnAspLysProTyrGlyGlnValValAlaAspGlnGlnCysIleThrG

      2650                2670                2690
AAGGCGTGGTTGCAAGCGCTCTATTCGATAAAGCATTCCTTCAGCCCGGGGAAGAGaCCG
luGlyValValAlaSerAlaLeuPheAspLysAlaPheLeuGlnProGlyGluGluThrG

      2710                2730                2750
AACTTTATATTGTGCGGGATAAATTATTTAAAGAGCGTCAGACTCGTGTTACTACGCGCC
luLeuTyrIleValArgAspLysLeuPheLysGluArgGlnThrArgValThrThrArgP

      2770                2790                2810
CAAGCCTGATTAAAAGATAATATGAGAAAAGCTACTTTTCTTACAGTGATCTCGTTTGGT
roSerLeuIleLysArg    MetArgLysAlaThrPheLeuThrValIleSerPheGly
                     ORF5
      2830                2850                2870
TTAGTTGTCTTTTTGGGTACCAGTTATTACTACCAAACTAAGTATATCCCGAAAGGCACC
LeuValValPheLeuGlyThrSerTyrTyrTyrGlnThrLysTyrIleProLysGlyThr
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      2890                2910                2930
GTGAATATATTTCCTGATGTCCACCGACAGGGTGAGATCGTTGATGATGCCTTTGAGAAA
ValAsnIlePheProAspValHisArgGlnGlyGluIleValAspAspAlaPheGluLys

      2950                2970                2990
AGCACGATTGTTATAAGCGACCCAACGCTTGCGGATAAATTTGTTGGAAGTTCAACCGAG
SerThrIleValIleSerAspProThrLeuAlaAspLysPheValGlySerSerThrGlu

      3010                3030                3050
TCTGTCGGTCGGCATGAAGTTAATGTAATAAAAGAATTGGGCACGCAAAATAATGGTTCG
SerValGlyArgHisGluValAsnValIleLysGluLeuGlyThrGlnAsnAsnGlySer

      3070                3090                3110
AAATTTATTCATGATGAGTCTGATAAATCAGGTTTGACGGTTGGAGATAAATGGCCTAGG
LysPheIleHisAspGluSerAspLysSerGlyLeuThrValGlyAspLysTrpProArg

      3130                3150                3170
GCTGGTGAACCTTATATCGTGCCTCAGATGACAGAATACGAGCGGAATTTGAAAGTTAAG
AlaGlyGluProTyrIleValProGlnMetThrGluTyrGluArgAsnLeuLysValLys

      3190                3210                3230
CGATTCCAGCAACCAAAGAATGGAGTTAATAATGGACATTAAAAAGGCCTGGGAAAATAA
ArgPheGlnGlnProLysAsnGlyValAsnAsnGlyHis
                                  MetAspIleLysLysAlaTrpGluAsnLy
                                  ORF6
      3250                3270                3290
AACTGTCAGAATCTCAGTAATTGGTGCTGCATTGATGGTATTGATTGTTGTCATCAGTCA
sThrValArgIleSerValIleGlyAlaAlaLeuMetValLeuIleValValIleSerGl

      3310                3330                3350
ATCAATCTTTACCACACCAGTTAAAAAAGAGAAGAAAACCCAGAAAAAAGATATGCAAAC
nSerIlePheThrThrProValLysLysGluLysLysThrGlnLysLysAspMetGlnTh

      3370                3390                3410
TGGATTCCTTATTGATGATTCACAAATGAACAAGCTGAGTAATGAGGAAAGCCAGAAGAC
rGlyPheLeuIleAspAspSerGlnMetAsnLysLeuSerAsnGluGluSerGlnLysTh

      3430                3450                3470
TTATAACGAAATGGTAAGGCAGAACCGTATTGACCAGAACGCTGCnAAGGCTGACCGTGA
rTyrAsnGluMetValArgGlnAsnArgIleAspGlnAsnAlaAlaLysAlaAspArgAs

      3490                3510                3530
CAAAGCTGAAAAGGCTCAGCAAGAAAACAAAGCACAGATAGCTAGTCTTATTCGCAAGTT
pLysAlaGluLysAlaGlnGlnGluAsnLysAlaGlnIleAlaSerLeuIleArgLysPh

      3550                3570                3590
CAGCAACTCACTACGCAGTAGACTGATATGCAGACCTCAAGAAATGGTAATCGCAATCTC
eSerAsnSerLeuArgSerArgLeuIleCysArgProGlnGluMetValIleAlaIleSe
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      3610                3630                3650
GATGCAGGCGGACCACGCAACAACGTCAATGAACAAGCCCACAAAGCCATACCAGCTTAA
rMetGlnAlaAspHisAlaThrThrSerMetAsnLysProThrLysProTyrGlnLeuAs

      3670                3690                3710
TCCAAATGCAGCGGTGAACGGGGTTAGTTCTGGATACGCACCTATATCTCCGACCCGAAA
nProAsnAlaAlaValAsnGlyValSerSerGlyTyrAlaProIleSerProThrArgAs

      3730                3750                3770
CAGTCCAATGCGGACAATAACACAGAGTTCAATTAAGACAAACGGCACTGATGGTGTCAT
nSerProMetArgThrIleThrGlnSerSerIleLysThrAsnGlyThrAspGlyValIl

      3790                3810                3830
TCAGGTGATGCCGATATCAGAGAACCGGATCAGAGAGGGGCGCGAGGTTGTTGCGGGAAG
eGlnValMetProIleSerGluAsnArgIleArgGluGlyArgGluValValAlaGlySe

      3850                3870                3890
TGAAAAGGCCCCAACGCGAACTATTCGCGGCGATGGGACTGCACCTGTTGATAGTAAAGC
rGluLysAlaProThrArgThrIleArgGlyAspGlyThrAlaProValAspSerLysAl

      3910                3930                3950
TCGTCATGCGGCCCGTAAGGATGAAATGTTTCTTCCAGCGACGTCAATTATTACTGGGGT
aArgHisAlaAlaArgLysAspGluMetPheLeuProAlaThrSerIleIleThrGlyVa

      3970                3990                4010
GCTAATTACCGGGCTGGAAGCTCCAACGAGTTTGTCCTCTAAAGCAGAACCGATGCCGGT
lLeuIleThrGlyLeuGluAlaProThrSerLeuSerSerLysAlaGluProMetProVa

      4030                4050                4070
TACCATGCGCATTAAAAAAGACATCATCATGCCTAACAATTACACGATGGATTTGCGTGA
lThrMetArgIleLysLysAspIleIleMetProAsnAsnTyrThrMetAspLeuArgAs

      4090                4110                4130
TTGTAATTTATTGGGTTCTGCTGTGGGGGATCTGGCATCTCAGCGTGCGTACATTAGGGC
pCysAsnLeuLeuGlySerAlaValGlyAspLeuAlaSerGlnArgAlaTyrIleArgAl

      4150                4170                4190
AACCTCCATATCGTGTGTCAACTCGAAAGGAAAAGCATTCGACGTAATAGTGGAAGCTTA
aThrSerIleSerCysValAsnSerLysGlyLysAlaPheAspValIleValGluAlaTy

      4210                4230                4250
TGCGGTTAGTGAAAATGACGGGAAAAATGGCATTCGTGGGAATCTGATAAGCAGAAATGG
rAlaValSerGluAsnAspGlyLysAsnGlyIleArgGlyAsnLeuIleSerArgAsnGl

      4270                4290                4310
AAATGCGATTGCAGGTTCTGCGTTTGCAGGAGGGCTGTCAGCATTGGCGGGGAGTCTGAG
yAsnAlaIleAlaGlySerAlaPheAlaGlyGlyLeuSerAlaLeuAlaGlySerLeuSe
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      4330                 4350                 4370
TCCATCAAAGGTGTCTTCGCTTAATATAGATCCAAACTCTACGGCACAATACCAATCGCC
rProSerLysValSerSerLeuAsnIleAspProAsnSerThrAlaGlnTyrGlnSerPr

      4390                 4410                 4430
AAACATAGGTGCATTAGGAGCGCTGGCCGGAGCCGGTGCAGCTCAGGGGGGTCTAAACCG
oAsnIleGlyAlaLeuGlyAlaLeuAlaGlyAlaGlyAlaAlaGlnGlyGlyLeuAsnAr

      4450                 4470                 4490
TCTTGTGGACTACTATACGTCAATAGCTGAGCAGCAGTGGCCAATTGTAGAAATTAGCCC
gLeuValAspTyrTyrThrSerIleAlaGluGlnGlnTrpProIleValGluIleSerPr

      4510                 4530                 4550
AGGGCGCCCAATTACTTTTGTCGTTCAAAAAGGGGCGACAATACCTACAAACCTGACAAG
oGlyArgProIleThrPheValValGlnLysGlyAlaThrIleProThrAsnLeuThrSe

      4570                 4590                 4610
CCGCTGAGAATAATTATGGAAAATAACAACGACAATGAATCACCAAAAAACAGTGAGTCT
rArg           MetGluAsnAsnAsnAspAsnGluSerProLysLysSerGluSer
               ORF7
      4630                 4650                 4670
GCTGATAGCTTAAATATCGACGCGGCTAAACCGGTTGGGGGTATACGTACTACCAAAAAA
AlaAspSerLeuAsnIleAspAlaAlaLysProValGlyGlyIleArgThrThrLysLys

      4690                 4710                 4730
GTAACTTTAGAGATCGATATCGAGAAAACTCTTAAGTATCTGATGGTCGCAGGCCTGGCA
ValThrLeuGluIleAspIleGluLysThrLeuLysTyrLeuMetValAlaGlyLeuAla

      4750                 4770                 4790
GTTCTTATCGTGATCTATgGATACAAGGGTGGACGTTTTGTATATGACAACTTCAAAGCA
ValLeuIleValIleTyrGlyTyrLysGlyGlyArgPheValTyrAspAsnPheLysAla

      4810                 4830                 4850
ATGTCGCAACCGGCCTACCAGATTGCCGTGCTGGATATGCAAACTTTAAGAAAAGTATTT
MetSerGlnProAlaTyrGlnIleAlaValLeuAspMetGlnThrLeuArgLysValPhe

      4870                 4890                 4910
AATAAGCAAAATCCGGTGTTGGATTCGCAGCAATCCAAAACTAACTTTGAAAATTATTTT
AsnLysGlnAsnProValLeuAspSerGlnGlnSerLysThrAsnPheGluAsnTyrPhe

      4930                 4950                 4970
AAGGCATTAATGAAGGTTTACCGTGAACGTGGCTATTTGGTTATTGATGCATCCTTGGCA
LysAlaLeuMetLysValTyrArgGluArgGlyTyrLeuValIleAspAlaSerLeuAla

      4990                 5010                 5030
GTAACTATTCCAGAGAATGTTGAAATAGTCTCTTATATCGACCTTGGTGGAAGTTTTGGT
ValThrIleProGluAsnValGluIleValSerTyrIleAspLeuGlyGlySerPheGly
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      5050                  5070                  5090
GAGGTACAATCTCTTCCAGGCGAACCTAAAGAAAGTGAGATTCGATAATTGAAAAAGTTA
GluValGlnSerLeuProGlyGluProLysGluSerGluIleArg

      5110                  5130                  5150
CTTTTGTCTCTGCTGATTTCCATTACCTCAGCGTGCCCGCCGCTTACTCTGGCTGTTGAA

      5170                  5190                  5210
AATGTATTAAACAATCGTGATGCCGCTGCGTTAATGGAAAAGGCAAAAGAAGGTGGAGTA
                                  MetGluLysAlaLysGluGlyGlyVal
                                  ORF8
      5230                  5250                  5270
TCAACGAAACCTTTAGACGGTGTATTGGCGAAAATGAAAAGCTATAAGCCTAAAGATGCT
SerThrLysProLeuAspGlyValLeuAlaLysMetLysSerTyrLysProLysAspAla

      5290                  5310                  5330
ATTTACATCCCTACTGGCGGTCTTTATTTGTTCCAGGATGAACGTTCTCAGTTGATGGCG
IleTyrIleProThrGlyGlyLeuTyrLeuPheGlnAspGluArgSerGlnLeuMetAla

      5350                  5370                  5390
GTGACCACTGACGGACGGTATACAATCACGGGGGGGAGCGTCATGGACATTCTTCAGAGA
ValThrThrAspGlyArgTyrThrIleThrGlyGlySerValMetAspIleLeuGlnArg

      5410                  5430                  5450
AAGTCAGTGTTATCAGTAGAGGATATACGAAAGTCGTTCTTTATTAATTTAGACGACGCC
LysSerValLeuSerValGluAspIleArgLysSerPhePheIleAsnLeuAspAspAla

      5470            ClaI.
CCTTTCCCTCTTGAAACTGTGGCATCGAT
ProPheProLeuGluThrValAlaSer
```

In order to precisely locate the exact mini-Tn10 insertion point, several of the Tra$^-$ mutants with insertions in either *Sal*I-G or *Sal*I-F were sequenced. In each case, the *Sal*I fragment harboring the insertion was subcloned to pUC21. Restriction endonuclease mapping of the cloned *Sal*I fragment allowed an approximate assignment of transposon insertion location, which was then confirmed by nucleotide sequence analysis. In order to determine the exact location of insertion, each subcloned fragment was sequenced using two unique primers with homology to the ends of the mini-Tn10 transposon. Insertion sites for mutants mapped in this manner are indicated in Figure VII-4.

After assembling tracts of sequence data from both the *Sal*I-F and *Sal*I-G fragments, the data were submitted for database comparison with both nucleotide and protein sequence databases. The BLAST and BLASTX programs, supplied by the National Center for Biotechnology Information were used to compare submitted sequences to non-redundant nucleotide and protein databases, respectively. In each case, default parameters were passed to the BLAST server. No significant homology was detected with any nucleotide sequence data used in the comparison. However, significant homology was detected at the amino acid level with the TraB, TraC, and TraL proteins encoded by the F plasmid. The homology was located in the *Sal*I-F fragment, and is indicated in Figure VII-6. All three open reading frames (TraB, TraC, and TraL homologs) are in the same orientation. In fact,

most significant open reading frames located within the S*al*I-F sequence deduced thus far are located in this orientation. Very few open reading frames of significant length could be found in the opposite orientation within the sequence. Comparisons of basic polypeptide parameters (molecular weight, number of residues, and isoelectric point) of TraB and TraL with homologous R27 proteins are shown in Table VII-3. Alignments with their R27 analogs are depicted in Figure VII-7. No significant homology was detected at the amino acid level with deduced translation products encoded in the S*al*I-G sequence submitted for comparison.

Table VII-3 Comparison of physical properties of F Tra proteins and R27 homologs

Protein[a]	Amino Acid Residues[b]	Molecular Weight	Isoelectric Point
TraB (F)	475	50,460	5.18
ORF6 (R27)	455	48,956	10.46
TraL (F)	91	10,379	10.64
ORF2 (R27)	116	13,642	10.73

[a]ORF designation refers to open reading frames identified in the sequence depicted in Figure VII-5.

[b]Physical data for F proteins taken from reference 41. Physical data for R27 proteins were calculated using the Genetics Computer Group program PEPTIDESORT

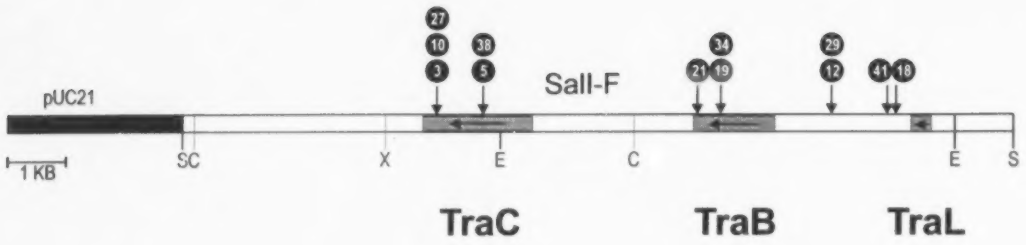

Figure VII-6 Location of open reading frames with homology to F Tra proteins

Nucleotide sequence, translated in all six reading frames, was compared with protein sequences deposited in the NCBI non-redundant protein database using the BLASTX program supplied by NCBI. Significant homology at the amino acid level was reported with the F transfer proteins TraB, TraC, and TraL. The location of nucleotide sequence encoding this homology is indicated by shaded boxes in the map above. The arrows indicate direction of transcription/translation. Mini-Tn10 insertion sites producing a Tra⁻ phenotype in R27 are shown above, as described in Figure VII-4. Restriction sites are as follows: S - *Sal*I, C - *Cla*I, X - X*ba*I, E - E*co*RI.

Figure VII-7 Alignment of R27 ORFs with F Tra proteins

Alignments are shown for ORF2 and TraL above, and ORF6 and TraB below. In the case of ORF6 and TraB, only the homologous portion of the sequence is shown (the C-terminal portion of the proteins). Solid lines (|) connecting sequences indicate amino acid identities, while dots (:) indicate amino acid similarities. Alignments were generated with Genetics Computer Group program FASTA.

Alignment of R27 ORF2 with F TraL

```
                10        20        30        40        50        60
ORF2    MRGIFPLSGVFMTSQYEIPPHTYRFPYRINMPLLILFWDAKQLGITFVTIASGNIFDFFI
                                  ||||  :: :   :    ::|  : : |: | : : ::
TraL                MSGDENKLKKYRFPETLTNQSRWFGLPLDELIPAAICIGWGITTSKYL
                               10        20        30        40

                70        80        90       100       110
ORF2    TSVVVAVVFWFAYKKAAEEGIRGKLKHKLWWYGFFPGKSVFSSRYFTDPFIRNLYSX
         :: :||: :|: ||  ::  :: |:: ::||
TraL    FGIGAAVLVYFGIKKLKKGRGSSWLRDLIYWYMPTALLRGIFSX
        50        60        70        80        90
```

Alignment of R27 ORF6 with F TraB

```
            220       230       240       250       260       270
ORF6    SEKAPTRTIRGDGTAPVDSKARHAARKDEMFLPATSIITGVLITGLEAPTSLSSKAEPMP
                                      ::|::|: :::||:| :| :|:::::::::|
TraB    QVTYQSVPVPNRIQRKVFTRNEGKQGPSLPYIPSGSFAKAMLIEGADANASVTGNESTVP
             190       200       210       220       230       240

            280       290       300       310       320       330
ORF6    VTMRIKKDIIMPNNYTMDLRDCNLLGSAVGDLASQRAYIRATSISCVNSKGKAFDVIVEA
        : :||:  : |||: | |  :| :  :| ||::|:|| :|: :|||:: :||::|: :::
TraB    MQLRITGLVEMPNSKTYDATGCFVGLEAWGDVSSERAIVRTRNISCLK-DGKTIDMPIKG
             250       260       270       280       290       300

            340       350       360       370       380       390
ORF6    YAVSENDGKNGIRGNLISRNGNAIAGSAFAGGLSALAGSLSPSKVSSLNIDPNSTAQYQS
        ::  :  |||||:|::: |||: | | |:::|: :  |  : :::|  ::: ::||:| :
TraB    HV--SFRGKNGIKGEVVMRNGK-ILGWAWGAGFVDGIGQ-GMERASQPAVGLGATAAYGA
                310       320       330       340       350

            400       410       420       430       440       450
ORF6    PNIGALGALAGAGAAQGGLNRLVDYYTSIAEQQWPIVEISPGRPITFVVQKGATIPTNLT
         ::  :|   |:||:::: : | |||:: |||  |:: |::|: :| | |:|
TraB    GDVLKMG--IGGGASKAA-QTLSDYYIKRAEQYHPVIPIGAGNEVTVVFQDGFQLKTVEE
        360       370       380       390       400       410

ORF6    SRX

TraB    MALERTQSRAEEDNPESPVPVPPSAESHLNGFNTDQMLKQLGNLNPQQFMSGSQGGGNDG
           420       430       440       450       460       470
```

Chapter VIII Discussion

This study represents an initial investigation at the molecular level of the mechanisms by which IncHI1 plasmids propagate. Plasmids of the H complex, and for that matter, of many other incompatibility groups, devote much of their coding capacity to functions that help to ensure their survival in a bacterial population. These include mechanisms of vertical transmission from one generation to the next (maintenance functions) and mechanisms of horizontal transmission from one host cell to another (transfer functions). The IncHI1 plasmid R27 was selected for this study as the prototypical member of the HI1 group.

A preliminary genetic and molecular analysis of the maintenance and transfer functions encoded by R27 was undertaken in this study. Comparison of these data with other plasmid systems has helped to elucidate the evolutionary relatedness of H plasmids with those of other major incompatibility groups.

A. RepHI1A - a minimal replicon of R27

Until recently, H plasmids have not been well characterized in terms of their replicative properties. An examination of the replicons encoded by these plasmids may assist in this, providing insight into the evolutionary relatedness of H plasmids with those of other Inc groups. A PolI-independent

replicon was isolated from R27 by subcloning an autoreplicative fragment from the S*al*I-E/F region of the plasmid. Based on observed specificity for plasmids of HI1 subgroup, and that this was the first replicon isolated to demonstrate such specificity, this replicon was designated RepHI1A, in keeping with nomenclature established for designating replicons of the F plasmids (16).

Initial testing of incompatibility traits of the minimal replicon failed to reveal any incompatibility between the parent plasmid, R27, and high-copy subclones of the replicon. This is unusual since similar (or in this case, identical) replicons typically exhibit strong incompatibility when co-resident in the same bacterial host, particularly when plasmid copy number is low (as in the case of R27). We were, however, able to demonstrate a potent incompatibility reaction between high-copy subclones of the replicon and a plasmid consisting of the minimal replicon ligated to a kanamycin cassette. This suggests that R27 harbors at least one additional functional replicon that is compatible with the replicon reported here. This second replicon (RepHI1B) has recently been identified, and molecular analysis of RepHI1B has shown it to be structurally similar to RepHI1A (45).

Examination of bi-directional deletions of the minimal replicon demonstrated that at least two separated regions within the replicon are capable of exerting incompatibility. Sequence data showed that these regions harbour multiple repeating oligonucleotides. Further subcloning

demonstrated conclusively that both sets of iterons within the replicon are responsible for the observed incompatibility of the replicon. This suggests that the iterons play a crucial role in the tightly regulated replication of this replicon. The presence of additional iterons *in trans* in large numbers completely shuts down replication of RepHI1A.

A dissection of the minimal replicon has shown that two basic elements are necessary for function. The *repA* gene encodes a *trans*-acting protein essential for activation of the origin. Elements upstream of the RepA coding sequence comprise the origin of replication itself and act *in cis*. We have defined a minimal origin of 230-bp, which contains two DnaA binding sites, multiple Dam methylase recognition sequences and four 19-bp iterons. Although further analysis is required to delineate the precise limits of the origin, as a separate unit, the four iterons are insufficient to provide a functional origin.

Expression of the RepA protein, required *in trans* for activation of the origin, appears to be tightly auto-regulated. The presence of RepA protein drastically reduces transcription from the *repA* promoter. Based on studies with related replicons such as P1, RepA likely binds to the iteron sequences, which may explain its autoregulatory function, since the *repA* promoter overlaps the origin iterons. Binding of RepA to these iterons may occlude RNA polymerase, preventing transcription.

The RepA coding sequence is not preceded by a sequence with homology to the 3' end of 16S rRNA, which would suggest poor translation initiation. However, some sequence similarity to a consensus Shine-Dalgarno sequence does exist near nt 470-473, some 24 bp upstream of the putative ATG start codon of the RepA coding sequence. A short, 21 bp open reading frame is present in the intervening sequence, whose stop codon (TGA) overlaps the start codon of the RepA coding sequence. Translational coupling of this "leader peptide" may provide a more efficient means of translation of the RepA gene.

These investigations have demonstrated that the iterons present downstream of the *repA* gene are not required for basic replicon function. However, they do appear to play an important role in copy number control, since deletion of all downstream iterons results in a substantial copy number increase. Studies of the closely related mini-replicon of plasmid P1 have shown that the iterons promote plasmid-plasmid pairing of like replicons, which appears to inhibit initiation of replication by steric hindrance (2, 103). This mechanism of copy control provides very tight control over copy number irrespective of RepA concentration.

The RepHI1A replicon appears to belong to a family of related replicons which have diverged from a common ancestral progenitor. Significant homology at the amino acid level is apparent between the Rep proteins encoded by the RepFIB replicon, the P1 replicon, and the replicon of

plasmid Rts-1. All of these replicons employ an iteron-binding scheme and all possess two set of iterons. The specificity of each replicon lies in the iteron sequence, which is unique to each replicon, such that all are compatible with one another. Conserved sequences in the proteins presumably reflect common function, such as interaction with host components of replication, while less highly conserved regions may reflect specific iteron interaction. A careful examination of this group of related proteins may lead to a directed analysis of functional domains within them.

B. Other maintenance elements of R27

In addition to RepHI1A, other known or suspected replication and maintenance elements have been discovered on R27. A partial replicon with homology to the RepFIA replicon of the F factor was previously identified and located on the R27 map (123, 153). The presence of this replicon was shown to be responsible for the one-way incompatibility observed between IncHI1 plasmids and the F factor, whereby co-introduction of an IncHI1 plasmid and the F plasmid into a common host bacterium always results in the loss of the F plasmid. In addition to this, Gabant *et al.* (45) isolated another PolI-independent replicon, RepHI1B, from the IncHI1 plasmid pIP522. In collaboration, we were able to map the RepHI1B replicon to R27, and discovered that it is located in the same vicinity as RepHI1A, far away from the RepFIA-like replicon. These results demonstrate the multireplicon

character of the IncHI1 plasmids. Plasmids of this group harbour at least three replicons: the RepFIA-like replicon previously described, and the two PolI-independent replicons, RepHI1A and RepHI1B described in this work.

Contrary to prediction, neither RepHI1A nor RepHI1B exhibit incompatibility with their parent plasmids, when cloned separately (45). Only when both replicons are cloned together (as in pDT2354 - see Figure IV-1) do they express incompatibility with R27. This suggests several important details concerning the replicative properties of IncHI1 plasmids. First, incompatibility is not observed between each of the cloned replicons and its parental plasmid due to the ability of one replicon to take over the replication of the plasmid if the other is in the presence of incompatible replicons. If, for example, a high copy clone carrying the RepHI1A replicon is introduced into a strain carrying R27, replication events initiating at RepHI1A should be severely restricted, or completely inhibited. However, our data implies that under these conditions, the RepHI1B continues to function, allowing the plasmid to replicate normally. This also suggests that each replicon functions independently of the other. When both RepHI1A and RepHI1B are suppressed simultaneously, incompatibility is observed.

The observation that pDT2354 (harboring complete RepHI1A and partial RepHI1B replicons) expresses incompatibility with R27 also implies that if other replicons are present within IncHI1 plasmids (such as the RepFIA-like replicon), they are incapable of stably replicating the plasmid. It

is interesting to note that although either RepHI1A or RepHI1B appears to be capable of stably replicating the entire plasmid without contribution of the second replicon, all IncHI1 plasmids examined retained both replicons. It may be that the carriage of multiple, independent replicons allows plasmids to co-exist with a broader range of competing plasmids within the bacterial host cell.

This hypothesis is supported by the observation that all IncHI1 plasmids also carry the partial RepFIA replicon, in addition to the two HI1 replicons. Despite the inconsequential contribution of this "accessory" replicon to the stable replication of the plasmid, all IncHI1 plasmids continue to maintain the replicon. Considering that plasmids of the F and HI incompatibility groups inhabit a very similar host range, it would seem that possessing a partial RepFIA replicon could potentially provide a competitive advantage to an HI1 plasmid, allowing it to easily displace F-like plasmids from a bacterial population. Further study is necessary to examine the advantage conferred to plasmids of harboring multiple functional replicons, if any.

Couturier *et al.* (29) cloned a fragment (pULB2436) from the IncHI1 plasmid TR6 that expresses incompatibility towards IncHI1 plasmids and has subsequently been used in replicon-typing of unclassified plasmids. Incompatibility tests showed that this *inc* region expresses incompatibility against representative plasmids of both the IncHI1 and IncHI2 subgroups,

and thus this region comprises the element or one of the elements that relates all plasmids of the IncHI group. It is of interest to note that although this region expresses incompatibility against both IncHI1 and IncHI2 plasmids, this is not accompanied by significant homology at the DNA level (29). This suggests a conserved function despite divergence of the DNA sequence. In light of the incompatibility expressed by this determinant, it has been designated IncHI1.

Mapping this determinant to the R27 genome revealed it to be located in the S*al*I-F fragment, near the RepHI1A and RepHI1B replicons. It is interesting to note that the IncHI1 determinant appears to be located within the previously defined Tra1 region of R27 (see Figure IV-2). In fact, closer analysis of the Tra1 region (reported herein) suggests that it may actually comprise at least two separate regions. The IncHI1 and RepHI1A determinants straddle one section of Tra1, while the remaining section(s) of Tra1 lie outside of this region (coordinates 160 to 20 on the R27 map).

Incompatibility loci are typically regions coding for plasmid maintenance elements such as control of replication or partitioning (105). Clones containing the IncHI1 locus but not the RepHI1A replicon are not autoreplicative, which suggests that this region is not involved in plasmid replication. We observed that clone pDT1233, which harbours both the RepHI1A replicon and the IncHI1 locus, is stable when transformed into a *polA* strain, which causes the plasmid to initiate replication solely from the

RepHI1A origin. Subclones containing only the RepHI1A replicon, without the Inc locus are unstable in the same background (Figure IV-1 and Figure IV-3). These data suggest that the IncHI1 locus may be involved in plasmid stability. Specifically, this region may encode a partitioning locus. Partition-mediated incompatibility has been described in other plasmid systems (170) and arises when the cloned partition site is introduced into a cell with the parent plasmid. The presence of the cloned partition site causes the partitioning machinery to randomly partition the parent plasmid, since it cannot distinguish the cloned fragment from the parental plasmid.

The DNA sequence of this IncHI1 region contains five direct repeats of 30-bp (46). Repeats have also been described in other partitioning sites (170). In these systems DNA repeats are located within a site that interacts with specific plasmid-encoded proteins to mediate an active distribution of the plasmid pool into daughter cells. Further molecular and genetic analyses are required to confirm that this IncHI1 locus is involved in the formation of such a partitioning complex.

C. Characteristics of the transfer system of R27

1. Complementation analysis

Complementation of R27 Tra$^-$ mutants with cloned fragments of R27 has enabled us to identify *trans* acting transfer functions in the two major

Tra regions of R27. The data suggests that there are at least two function defects represented in the Tra2 insertion mutants. In addition, two potential complementation groups have been recognized in the Tra 1 region. Two Tra$^-$ mutants in this region, pDT1180 and pDT1185, map in close proximity to one another, yet one can be complemented in trans while the other cannot. This would seem to signify the presence of two distinct complementation groups.

Preliminary testing for the establishment of an IncHI/IncHII complementation system has revealed that complementation of the IncHI transfer-defective mutants does occur, but is host dependent in its degree. Of four bacterial hosts used for complementation, *E. coli* K-12 J53-1 proved to be the most efficient. Evidently, the means by which the transfer mechanisms of these two plasmids interact involves certain host factors. Since the RecA$^+$ hosts tested were the most efficient for transfer of R27 Tra$^-$ mutants in the presence of the IncHII plasmid, it was suspected that transfer was occurring by recombination (or possibly transposition) of antibiotic resistance markers between the two plasmids. However, further investigation, both by genetic means and by direct analysis, suggested that in the majority of cases, recombination was not occurring.

The data suggest that the transfer systems of R27 and pHH1508a are related to some extent, despite the presence of little DNA homology between the two plasmids. This is not an unreasonable assumption, considering the antigenic and morphological relatedness of the conjugative pili expressed by

IncHI and IncHII plasmids. The considerable differences seen in complementation efficiency amongst the R27 Tra$^-$ mutants tested may reflect differences in function between the transfer apparatus of the two plasmid groups. Of particular interest is the observation that in two of the hosts tested (HB101, and JE2571), the presence of pHH1508a appears to suppress the transfer of a wild-type R27 plasmid at 26°C. This may indicate competition for limiting host factors involved in transfer. Since neither of these hosts is particularly well-characterized in terms of precise genotypic differences from wild-type *E. coli* strains, it is impossible to determine which host mutations appear to be conferring this competition phenotype. Numerous *E. coli* chromosomal loci have been shown to affect transfer of the F factor, including *cpxA*, *cpxB*, *sfrA*, *sfrB (rfaH)*, and *fexB* (131, 132), and it appears from these data that others may also be involved in H plasmid transfer.

The transfer behaviour of R27 and R27 Tra$^-$ mutants in the presence of pHH1508a discloses some information regarding the relatedness of the transfer mechanisms of these two plasmids. In particular, the successful transfer of R27 and several Tra$^-$ mutants at 37°C in two of the hosts tested demonstrates that the temperature sensitivity of the transfer mechanism of this plasmid can be at least partially compensated for *in trans* by pHH1508a. Several of the Tra$^-$ mutants in particular transferred at frequencies

approaching that of the IncHII plasmid itself (approx. 1.0 x 10^{-2} transconjugants per recipient - data not shown) when tested at 37°C.

2. Temperature-sensitivity of transfer

Perhaps the most interesting characteristic of the IncHI plasmids is their temperature-sensitive transfer system. Although plasmids of the IncM group have been reported to express temperature-sensitive transfer (67), this has not been consistently reproducible (24, 80). Aside from this, no other plasmid group has been reported to possess a conjugation system with these characteristics. Previous studies have shown the pre-incubation temperature of the recipient culture to have little effect on transfer efficiency of HI plasmids, while the incubation temperature of the donor and mating cultures is critical (147). It was also demonstrated that *E. coli* cells harboring a de-repressed derivative of R27 do not produce conjugative pili at 37°C (81). These data suggest that HI plasmids are unable to transfer at higher temperatures due to an inability to produce conjugative pili.

The inhibitory effect incubation at 37°C has on the conjugation apparatus of R27 likely involves a relatively complete suppression of the synthesis of required components, since transfer of a mating culture from 37°C to 28°C results in the appearance of transconjugants at least two hours after the shift. In contrast, matings carried out at 28°C, with preincubation of donor and recipient cultures at the same temperature produce

transconjugants within 15 min. This shows a lag phase of at least 90-100 min. from a temperature shift until the transfer apparatus becomes functional.

Brief exposure to inhibitory temperatures prior to conjugation drastically reduces transfer frequency in a one hour mating. A direct logarithmic relationship exists between time of exposure and the inhibition of transfer, with a reduction rate of approximately 27% per min (i.e. 27% drop in transfer efficiency per minute of exposure at 37°C). In addition, exposing a mating culture to inhibitory temperatures for brief periods of time in the first 30 min. of a one hour mating also produces a dramatic decrease in transfer frequency of the same magnitude. This suggests that exposure to 37°C causes a rapid and prolonged suppression of the transfer system of R27. The rapid response may indicate that a pre-formed component in the transfer apparatus is affected by the temperature shift. It seems unlikely that a transcriptional control mechanism could exert an effect so quickly, although this also remains a possibility.

A mating culture that is shifted to 37°C within the first 5 min. of the mating fails to produce any detectable transconjugants. This demonstrates that the inhibitory effect of a temperature shift on the donor can interrupt a mating in progress. Comparison of mating cultures that are physically disrupted at various time points in a one hour mating, to a similar culture that is shifted to 37°C at various time points shows a time lag of

approximately 5-7 min. in observed mating efficiency (see Figure V-2). This suggests that the last 5-7 minutes of a transfer event are not susceptible to the effects of a temperature shift.

Mating aggregates form as an early step in the transfer process. It has been demonstrated that temperature shifts to 37°C prevent the formation of mating aggregates amongst donors harboring a derepressed derivative of R27 and an appropriate recipient (82). This effect is seen despite the presence of conjugative pili on the donor cells. This observation, taken with those above, suggests that temperature shifts immediately and permanently impair the ability of donor cells to successfully complete these early steps in conjugation.

Although temperature-shift experiments suggest a rapid and profound effect on mating cultures when exposed to temperatures above 35°C, the gross morphology of intact pili does not appear to be affected by incubation at these temperatures. Maher *et al.* incubated H pili at 37°C for periods up to 30 min. without any noticeable change in pilus morphology (82). If elevated incubation temperatures have any effect on the H pilus, it must be relatively subtle with respect to morphological changes in pilus structure.

D. Characteristics of the conjugative pili encoded by R27

Very little is known about the conjugative pili elaborated by H plasmids. Although morphologically similar to F pili, they are antigenically

distinct from conjugative pili produced by plasmids of other incompatibility groups. Previous attempts at characterizing the protein components of H pili have been relatively unsuccessful, largely due to the lack of an H plasmid that produces adequate numbers of conjugative pili for analysis. With the recent isolation of transfer-derepressed derivatives of R27 and other H plasmids, further analysis of H pili has become possible.

Developing a reliable method of isolating H pili proved to be somewhat challenging. Although PEG has been used successfully in the isolation of PAK and PAO pili from *Pseudomonas* strains, PEG concentrations up to 3% (w/v) failed to produce any detectable precipitation of H pili from a crude preparation. Ammonium sulfate (15% w/v) was found to be useful in the precipitation of pili from solution, but the concentration was critical to obtaining a relatively clean pili preparation. Although subjecting H pili preparations to cesium chloride density gradient centrifugation provided very clean samples, this technique very frequently resulted in loss of the pili, probably as a result of precipitation. Therefore, the method ultimately chosen for pili isolation employed several rounds of precipitation using ammonium sulfate as the primary method of purification. This resulted in the least sample loss, and provided relatively clean preparations, as judged by SDS-PAGE analysis of the isolated pili.

One of the most notable features of purified H pili is the very small subunit size of the pilin monomer that comprises the pili. In fact, the pilin

protein was much too small to resolve on standard polyacrylamide gels, and could only be clearly discerned on a high-resolution gel system designed for separation of small proteins and peptides. At an apparent molecular weight of 3.0- to 3.2-kDa, the H pilin protein is, to the author's knowledge, the smallest reported pilin protein to date.

Amino acid analysis of purified H pili corroborated the SDS-PAGE analysis, and suggested a subunit size of 2.7- to 2.9-kDa, in close agreement with pilin size determined by SDS-PAGE analysis. Of note is the lack of numerous amino acids in the pilin protein, including Arg, His, Pro, Cys, Met, Tyr, and Phe, although a protein consisting of just 24-26 amino acids would be expected to lack some residues. The absence of tyrosine in the protein would explain its inability to be detected in the mini-Lowry assay, since this assay depends on the presence of tyrosine or tryptophan residues in the assayed protein. The amino acid composition of the protein reveals the presence of 7 hydrophobic residues in the R27 pilin, yet overall, the protein is slightly hydrophilic, according to the hydrophobicity values of Kyte and Doolittle (70).

Despite numerous attempts at determining the amino terminal amino acid sequence of the pilin protein, no sequence could be obtained. This suggests that the amino terminus of the protein is blocked to Edman degradation. This is not unusual for pilin proteins. Many of the F-like pilin proteins possess N-acetyl blocking groups at their amino termini (40). This

F-pilin monomer is acetylated by the action of the *traX* gene (83, 94), and it has been suggested that acetylation may provide increased stability to the protein in a natural setting, although unacetylated pilin can be incorporated into a functional pilus (41), and the unacetylated product appears to have comparable stability to the acetylated product in pulse-chase experiments (83). The precise nature of the blocking group at the amino terminus of H pilin remains to be determined.

The pilin doublet that frequently appeared on SDS-PAGE analysis of purified pili is difficult to explain. The second band was not visible in all pili preparations, and appeared in varying proportion to the main pilin band when it did appear. Pilin samples containing one or both bands could not be sequenced, suggesting that a blocked N-terminus is present in both species. It is interesting to note that only one band (corresponding to the smaller pilin species) ever appeared on Western blots of either purified pili, or whole cell protein, which suggests that the secondary pilin band is not immunoreactive with the polyclonal sera used in these experiments. The second pilin band migrates slightly more slowly and may represent some sort of post-translation modification to the main pilin monomer, or possibly a precursor protein. Alternatively, it could simply be an artifact or contaminant from the purification procedure. A slower-migrating species of F pilin has also been reported. The evidence suggests in this case that the alternative form of pilin likely arises as a modification of the mature pilin monomer (83).

Attempts at proteolytic digestion of the pilin protein were all unsuccessful. It is likely that H pilin is relatively resistant to the denaturing conditions used in the digestion procedures, which would prevent critical residues from being properly exposed to the protease. That treatment with the denaturants used in the digestion (8M urea, 6M guanidine-HCl) failed to produce any noticeable disruption to intact pili would lend support to this hypothesis. Indeed, the same resistance to denaturing agents has been observed with F pili (11). It is also possible that some digestion may have occurred, but at a level that was below the limits of detection.

A "mini-pilin" produced by *Aeromonas hydrophila* has recently been reported (56, 57). This pilin protein has a molecular weight of 4.6-kDa and is the sole constituent of an environmentally-regulated flexible pilus produced by some strains of *A. hydrophila*. The *Aeromonas* pilus is not the product of a conjugative plasmid, but rather is expressed from the *fxp* gene, located on a 7.6-kb non-conjugative plasmid (57). Morphologically, the pilus is similar to H pili, in that it is classified as thick and flexible. Of note, is the observation that these pili are produced in much greater abundance at 22°C than at 37°C when the organism is grown in TSB or TSA media (56). This pilus is also extraordinarily resistant to denaturants. The overall amino acid composition compares somewhat to that of the H pilin, with a notable lack of Cys or aromatic residues, although the *Aeromonas* pilin possesses four Met residues as well a single Pro residue, neither of which are present in H pilin.

The Fxp pilin shows no overt homology with any other reported pilin proteins or colonization factor antigens (57).

It would be most intriguing to speculate on a possible relationship between the *Aeromonas* flexible pilin and H pilin. *A. hydrophila* is able to exist as a free-living organism in aquatic environments, and is a common pathogen of fish and other poikilotherms. H plasmids have been shown to transfer to *Aeromonas* with relatively high efficiency at temperatures as low as 14°C (80). In addition, *Aeromonas* sp. carrying high molecular weight R-plasmids have been reported (26). Considering these findings, the compositional and size relationships of Fxp and H pilin, and the thermosensitive nature of IncHI pilus expression, it is conceivable that Fxp and H pilin may be derived from a common ancestor. This intriguing possibility awaits the elucidation of the amino acid sequence of the H pilin for confirmation.

After absorption to remove cross-reacting activity, polyclonal anti-H pili antisera was found to be useful in detecting mature pilin monomer in whole cell protein mixtures. Although specific pathogen-free rabbits were used for antisera production, *E. coli* is unfortunately not part of the specific pathogen-free description, and numerous *E. coli* proteins reacted very strongly with the antisera collected after immunization with purified H pili - very likely a result of the rabbit's prior exposure to the organism. Absorption of the antisera removed a considerable portion of the cross-reactivity, and

allowed relatively clear visualization of pilin protein in whole-cell protein extracts from *E. coli* carrying pDT1942. The small size of the pilin protein was certainly helpful in its detection by immunoblot.

It is notable that of several H plasmids tested, only derepressed derivatives of wild-type plasmids produced sufficient quantity of H pilin to be detectable by immunoblot. The natural repression of transfer observed in most HI plasmids thus appears to be associated with a severe reduction in pilin production. The absence of any detectable H pilin in strains harboring IncHII plasmids was unexpected. Pili produced by HI and HII plasmids are morphologically indistinguishable, cross-react antigenically (23), and mediate attachment of the same pilus-specific phages, pilHα and Hgal (81). In addition, HII plasmids are relatively derepressed with respect to transfer, in comparison to HI plasmids, and strains carrying HII plasmids produce sufficient conjugative pili to be visible by electron microscopy (approximately 1 pilus per 20 cells). Because of this, it was expected that HII plasmids would produce a detectable pilin protein when assayed by immunoblot. However, no distinct band could be seen on immunoblots. It is possible that the HII pilin is considerably larger than the HI pilins, and as such, could be obscured on immunoblots. Alternatively, the HII pilin may not be antigenically reactive with the polyclonal sera used in this study, or the quantity present in whole-cell protein preparations may be below the limits of detection. Although HII plasmids are relatively derepressed for transfer in

comparison to HI plasmids, there is at least a 20-fold deficiency in pilus production by these plasmids when compared to the derepressed derivatives of the HI plasmids. This deficit in pilus production may also be reflected in synthesis of the pilin monomer.

Both HI2 plasmids that produced detectable amounts of H pilin (R478-drd and R477-1-drd) produced a pilin protein that migrated slightly more slowly in acrylamide gels than the pilin produced by R27. The amino acid analysis of R477-1 pili suggests that the HI2 pilin may contain one or two additional amino acid residues, and has a slightly different composition, being somewhat more hydrophobic overall than the HI1 pilin. Despite these differences, the HI1 and HI2 pilin proteins are obviously highly homologous, adding to the growing pool of evidence that shows IncHI1 and IncHI2 plasmids to be very closely related, despite the presence of relatively little DNA homology.

Western blot analysis showed conclusively that pilin monomer is not detectable when the culture is incubated at 37°C. This certainly explains why conjugative pili are not produced at this temperature, and suggests that either the biosynthetic components involved in pilin synthesis are inactivated at this temperature, or that the pilin monomer is itself unstable at this temperature. The long latency period between a shift in temperature from 37°C to 28°C and the appearance of pilin monomer lends support to the former conclusion. If the disappearance of pilin at 37°C was simply due to

instability of the protein at this temperature, it would be expected to reappear relatively rapidly after a shift in temperature. However, pilin protein does not appear for at least 90 min. after a temperature shift. This suggests a relatively complete shut-down in the pilin biosynthetic apparatus at 37°C.

A temperature shift from 28°C to 37°C produces a gradual decline in steady-state pilin levels over a period of several hours. It is impossible to say with this data whether new pilin synthesis ceases rapidly after such a shift in temperature. It is unfortunate that pulse-chase experiments failed to provide any insight in this area. The failure of the pulse-chase experiments may be due to a slow rate of synthesis of mature H pilin, or possibly to inadequate incorporation of radiolabelled amino acid into the pilin protein. The growth media used in the pulse-chase experiments may have contributed to this effect. H plasmid transfer occurs at much lower frequency in defined media such as those used in pulse-chase experiments, compared to matings in rich, undefined media (personal observation). This effect may be due to poor expression of transfer genes under these conditions.

Attempts at locating the coding sequence of R27 involved in pilin synthesis by examining proteins of strains harboring cosmid clones of the plasmid showed that none of the regions examined were capable of encoding high-level synthesis of mature H pilin. This would suggest that genes involved in pilin synthesis may be widely scattered throughout the plasmid,

or that critical components of pilin biosynthesis are encoded in the small unclonable region of R27 (see Figure I-2).

E. Mutagenesis and sequence of Tra genes encoded by R27

Although transposon insertion mutagenesis of R27 had previously been undertaken in order to characterize regions of the plasmid involved in transfer, the original mutagenesis was carried out using the wild-type, transfer-repressed form of R27. Because of this, none of the Tra$^-$ mutants generated could be characterized with respect to effects on H pilus biosynthesis. In this study, a similar effort was undertaken, using a mini-Tn10 transposon to mutagenize a derepressed derivative of R27. In this way, it was hoped that further characterization of the effects of the transposon insertions would be possible.

Mapping of the 40 Tra$^-$ insertion mutants revealed a distribution comparable to that delineated in previous studies, which had defined the two major transfer regions of the plasmid, Tra1 and Tra2. Taken with previous data, the insertion sites reported here suggest the Tra2 region to encompass approximately 15-kb extending from coordinates 99 - 114kb on the R27 physical map, while the Tra1 region covers a much larger area of approximately 57-kb extending from coordinates 163 - 38kb. Insertions in Tra1 are widely scattered throughout the region, and large gaps with no insertions are present, suggesting that Tra1 may in fact represent several

smaller Tra regions interspersed with other coding sequence of uncharacterized function. The presence of the IncHI1 locus between two regions shown to be involved with transfer lends support to this. In addition, no other plasmid system has been shown to possess coding sequence devoted to transfer functions that is as extensive as the sequence in R27 would be if the entire Tra1 region was contiguous. For example, the IncF plasmids carry approximately 33-kb of sequence devoted to transfer functions (41), while the IncP plasmids have two Tra regions totalling approximately 25-kb in size (109). It would seem unlikely that H plasmids would have more than twice this amount of coding sequence involved in transfer. If the transfer regions of R27 are distributed in several different sections of the plasmid, it would certainly represent one of the most highly genetically dispersed transfer systems, in comparison to those of other plasmids, which are more typically present in one or two distinct and contiguous sections.

Very few of the mini-Tn10 insertions had any noticeable effect on pilin synthesis. Even in the four mutants where an effect on pilin synthesis was observed, only one mutant failed to produce any detectable pilin. This suggests that only a limited number of genes are directly involved in the synthesis of mature pilin monomer. This mirrors the pilin biosynthesis of the F plasmid, where only three genes are directly involved in pilin synthesis, *traA*, *traQ*, and *traX* (43, 83, 94). The majority of the genes in the F Tra region are not directly involved in pilin biosynthesis.

It was initially suspected that insertions with mini-Tn10 could have polar effects on pilin biosynthesis, but the limited number of pilin mutants observed suggests this not to be the case. It is also possible that genes directly involved with pilin synthesis are located near the 5' ends of *tra* operons in R27, which would reduce the likelihood of insertions exerting polar effects on these genes. In the F plasmid, the *traA* gene, which encodes the propilin subunit, is located close to the 5' end of the main *tra* operon.

All of the insertion mutants which reduced, but did not eliminate, production of H pilin were located in the S*al*I-F fragment of R27. In fact, a large number of insertions were located in this fragment, a pattern which has been observed in previous studies using transposons Tn5, Tn7, and TnLacZ (a derivative of Tn5). It is possible that this region represents a hot spot for transposon insertion, but considering the common trend using the unrelated transposons Tn5, Tn7, and Tn10, it seems more likely that this region harbors a concentration of critical transfer-related functions, including genes whose function may be directly or indirectly involved in pilin biosynthesis.

The only insertion to completely eliminate detectable pilin synthesis (mutant #9) was surprisingly located a considerable distance away from the majority of other insertion sites in the Tra1 region. Indeed, this insertion was the only one located in the S*al*I-G fragment. The insertion may be in a small operon, possibly encoding only a single gene, which is apparently involved in pilin biosynthesis. A small operon size may explain the lack of

insertions located in this area, and that previous transposon insertion mutagenesis studies have failed to identify this specific region as contributing to transfer.

Surprisingly, most of the insertions reduced or eliminated sensitivity to the H specific phage used in the spot test. The notable exceptions included insertions in the S*al*I-H1, S*al*I-H2 and S*al*I-N fragments, none of which had any impact on phage sensitivity. This suggests that these regions do not encode genes whose function affects pilus biosynthesis. However, insertions in every other Tra region either greatly reduced or eliminated phage sensitivity, implying some involvement with pilus expression. Overall, the data suggest that large regions of the Tra1 area and a small section of Tra2 encode genes whose action affect the production of H pili. The majority of Tra2 and the S*al*I-H1 region of Tra1 likely encode genes whose products are linked to other transfer functions, such as signalling, origin nicking, and DNA transport during conjugation.

Nucleotide sequence analysis of the S*al*I-F fragment of R27 has revealed the presence of open reading frames with the potential to encode proteins with significant homology to the TraB, TraC, and TraL proteins of the F plasmid. All of these proteins are involved in pilus biosynthesis, although their specific contribution to this process is as yet not understood. It is interesting to note that homologs of these proteins have also been discovered in plasmids of the N, P, and W incompatibility groups, as well as

the Ti plasmid and the Ptl operon of *Bordetella pertussis*. This family of proteins is apparently involved in the secretion of macromolecules (64, 164).

The orientation and close spacing of the open reading frames within the nucleotide sequence reported here suggests that they may be part of an operon structure, transcribed from a common promoter further upstream. Most other plasmid transfer genes previously reported have also been shown to occur in operon clusters. All of the orfs in R27 with identifiable homology to F Tra genes are organized in the same relative order as in the F plasmid (*tra*L→*tra*B→*tra*C). However, homology with the intervening genes present in the F plasmid (*tra*E, *tra*K, *tra*P, *tra*V, *tra*R) cannot be detected in the sequence analyzed from R27. The gene encoding the pilin subunit in the F plasmids, *tra*A, is located immediately upstream of *tra*L. However, the orf upstream of the *tra*L homolog in R27 contains no homology with TraA, and the amino acid composition of the C-terminal region of the polypeptide it encodes has no similarity with the amino acid composition of H pilin. Unfortunately, it appears not to be the elusive H pilin gene.

F. Future studies in H plasmid genetics

It is obvious from these studies that much remains to be uncovered with respect to H plasmid genetics. This work has clearly demonstrated that HI1 plasmids are evolutionarily related to those of the IncY, IncT, and IncF groups by way of the two replicons, RepHI1A and RepHI1B, carried by all

HI1 plasmids. Characterization of the replicons carried by IncHI2 and IncHI3 plasmids would help to clarify the relationship between plasmids of the three HI subgroups, and possibly allow the reassignment of MIP233, the sole member of the HI3 group, to a different incompatibility group.

Stability and host killer systems have yet to be described in plasmids of the H complex. As unit copy plasmids, they must encode partitioning loci in order to maintain stability in their bacterial hosts. The IncHI1 locus identified in this study shows promise as a stability locus; further work will help to determine the precise role this locus plays in plasmid maintenance.

Although this and previous studies have shown regions of R27 involved with transfer to fall broadly in two large segments, it appears likely that the plasmid possesses several smaller Tra regions. Further sequencing and mutagenesis will enable the precise limits of the Tra regions to be deduced. Additional efforts at determining the N-terminal amino acid sequence of the pilin protein would help to characterize the H pilus, as well as assist in locating the pilin gene. Although this study has shown that temperature-induced inhibition of the IncHI transfer system is rapid and involves suppression of pilin synthesis, transcriptional and translational analysis of *tra* genes and Tra proteins may shed additional light on the basis of the temperature-sensitivity of the IncHI transfer system.

It is evident from this study that the transfer system of R27, and likely most of the IncHI plasmids, shares a common ancestry not only with F, but

with broad host range plasmids of several other incompatibility groups as well. Further analysis of this group of plasmids will serve to more accurately characterize this shared ancestry.

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Appendix 1 Media & Buffer Components

A Medium

K_2HPO_4	10.5 g
KH_2PO_4	4.5 g
$(NH_4)_2SO_4$	1.0 g
Na citrate•$2H_20$	0.5g

per litre, sterilized by autoclaving, then the following added:

0.4% glucose
1 μg/ml vitamin B1
1 mM $MgSO_4$

M9 Medium

Na_2HPO_4	6.0 g
KH_2PO_4	3.0 g
NaCl	5.0 g
NH_4Cl	1.0 g

per liter, sterilized by autoclaving, then the following added:

1 mM $MgSO_4$
0.2% glucose
0.1 mM $CaCl_2$

PBS (Phosphate Buffered Saline)

Na_2HPO_4	11.5 g
NaH_2PO_4	4.58 g
NaCl	8.5 g

per litre, autoclave to sterilize

TBS (Tris Buffered Saline)

Tris, pH 7.2	12.1 g
NaCl	8.5 g

per litre, autoclave to sterilize

Appendix 2 Amino Acid Abbreviations and Hydrophobicity Values

Amino Acid Abbreviation			Hydrophobicity
1 letter	3 letter	Amino Acid	Value[a]
A	Ala	Alanine	1.8
C	Cys	Cysteine	2.5
D	Asp	Aspartic Acid	-3.5
E	Glu	Glutamic Acid	-3.5
F	Phe	Phenylalanine	2.8
G	Gly	Glycine	-0.4
H	His	Histidine	-3.2
I	Ile	Isoleucine	4.5
K	Lys	Lysine	-3.9
L	Leu	Leucine	3.8
M	Met	Methionine	1.9
N	Asn	Asparagine	-3.5
P	Pro	Proline	-1.6
Q	Gln	Glutamine	-3.5
R	Arg	Arginine	-4.5
S	Ser	Serine	-0.8
T	The	Threonine	-0.7
V	Val	Valine	4.2
W	Trp	Tryptophan	-0.9
Y	Tyr	Tyrosine	-1.3

[a]Kyte and Doolittle, 1982 (70).